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ORIGINAL PAPER

The association between RAD18 Arg302Gln polymorphism and the risk of human non-small-cell lung cancer

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Abstract
Purpose The repair enzyme RAD18 plays a key role in the post-replication repair process in various organisms from yeast to human, and the molecular function of the RAD18 protein has been elucidated. Single nucleotide polymorphism (SNP) of arginine (Arg, CGA) or glutamine (Gln, CAA) at codon 302 is known on RAD18; however, the association between the SNP and the risk of any human cancers including non-small-cell lung cancer (NSCLC) has not been reported. We therefore investigated the relationship between the polymorphism and the development and progression of human NSCLC.
Methods The study population included 159 patients with NSCLC and 200 healthy controls. The SNP was genotyped by polymerase chain reaction with the confronting two-pair primer (PCR-CTPP) assay. Genotype frequencies were compared between patients and controls, and the association of genotypes with clinicopathological parameters was also studied.
Results The Gln/Gln genotype was significantly more frequent in NSCLC patients (20.7%) than in healthy controls (11.5%)($P = 0.003$). The increased risk was detected in NSCLC patients with the Gln/Gln genotype [Odds ratio (OR) = 2.63, 95% confidence interval (CI)=1.38–4.98]. As to the relationship of the SNP with clinicopathological parameters of NSCLC, significantly higher risks were detected in lung squamous cell carcinoma (LSC) (OR = 4.40, 95% CI = 1.60–12.1).
Conclusions Our results suggested that Gln/Gln genotype of the RAD18 SNP has the increased risk of NSCLC, especially of LSC. This is the first report to provide evidence for an association between the RAD18 Arg302Gln polymorphism and human NSCLC risk.

Keywords SNPs · RAD18 · Non-small-cell lung cancer (NSCLC) · Cancer predisposition

Abbreviations

LAD	Lung adenocarcinoma
LSC	Lung squamous cell carcinoma
NSCLC	Non-small-cell lung cancer
OR	Odds ratio
PCNA	Proliferating cell nuclear antigen
PCR-CTPP	Polymerase chain reaction with the confronting two-pair primers
PRR	Postreplication repair
SNP	Single nucleotide polymorphism

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Introduction

DNA in living cells is damaged by environmental damaging agents and mutagens, such as UV light and mutagenic chemicals (Hoeijmakers 2001). DNA damage must be repaired by

DNA repair systems. However, when the DNA repair systems are stalled or saturated, and such DNA damages are thus not removed before the onset of DNA replication, single-stranded gaps are generated. These gaps will be filled by the postreplication repair (PRR) system. The RAD6 pathway is known to be central to PRR (Lawrence 1994) and RAD6 epistasis group proteins, such as RAD5, RAD18, RAD30, MMS2 and UBC13, are all involved in the pathway. In this pathway, RAD18 and RAD6 are two of the most important proteins and play a key role. RAD18 is a single-strand DNA binding protein with a RING finger domain, and has ubiquitin-ligating enzymes (E3) activity (Joazeiro and Weissman 2000). RAD6 is an ubiquitin-conjugating enzyme (E2) in the proteasome protein degradation system (Sung et al. 1990, 1991b; Wood et al. 2003). RAD18 forms a tight complex with RAD6 (Bailly et al. 1994, 1997a; b). Although RAD6 interacts with several ubiquitin-ligating enzymes (E3), the interaction with RAD18 is essential for carrying out PRR (Wood et al. 2003; Bailly et al. 1994; Dohmen et al. 1991; Sung et al. 1991a).

RAD18 knockout cells of mouse embryonic stem cells (Tateishi et al. 2003) and of chicken DT40 cells (Yamashita et al. 2002) were hypersensitive to various DNA-damaging agents and showed defective PRR. Genomic instability of these cells was demonstrated by increased rates of the sister chromatid exchange and integration of exogenous DNA (Tateishi et al. 2003; Yamashita et al. 2002). RAD18 contributes to the maintenance of genomic stability through PRR and dysfunction of RAD18 increases the frequency of homologous recombination as well as illegitimate recombination (Shekhar et al. 2002). Furthermore, dysfunction of RAD18 is thought to lead to the development of cancer (Friedberg 2003).

The genetic polymorphisms of DNA repair genes have been analyzed to determine susceptibility to several cancers, including lung (Ito et al. 2004; Ryk et al. 2006), colorectal (Yamamoto et al. 2005), breast (Costa et al. 2006), head and neck (Huang et al. 2005), bladder cancer (Zhu et al. 2007) and leukemia (Bolufer et al. 2006). The *RAD18* gene is known to have a single nucleotide polymorphism (SNP) at codon 302, encoding either arginine (Arg, CGA) or glutamine (Gln, CAA), as known as rs#373572 in the dbSNP; NCBI Reference SNP (refSNP) Cluster Report. In the present study, we found a significant correlation of the SNP with NSCLC. This is, to our knowledge, the first report providing evidence for an association between the RAD18 Arg302Gln polymorphism and human NSCLC risk.

Materials and methods

Subjects

We studied frozen specimens of 159 cases stored at −80°C obtained from Japanese patients with primary NSCLC treated by curative intent surgical resection in Okayama University Hospital (Okayama, Japan), after acquiring informed consent from each patient, between 1994 and 2003. The case groups consisted of 105 lung adenocarcinomas (LAD), 48 lung squamous-cell carcinomas (LSC), 3 adeno/squamous-cell carcinomas and 3 large cell carcinomas (107 men, 52 women; mean age 66.2 years). The clinical stage and pathological grade in most patients were confirmed by operation and pathology. The clinical staging and histological classification of cancers were defined according to the criteria of UICC Tumor-Node-Metastasis Classification of Malignant Tumors (TNM), sixth edition, 2002, (ICD-O C34 for lung). For the controls, each of the 200 healthy controls we analyzed was selected by computer-aided randomization among five individuals matched in smoking habit, gender and age (within 5 years) for each lung cancer patient, all of which were from the subjects of cohort studies on a Japanese general population older than 40 years of age in a town near the Saitama Cancer Center. A population of this town has increased because of a population influx from other areas, with a social increase rate of about 5% every year for 15 years. Informed consent was obtained from all cases and controls concerned. This study was approved by The Bioethics Committee of Okayama University Medical School.

DNA extraction

Genomic DNA of 159 patients was isolated from the noncancerous region of the resected specimens or from the mononuclear cells of the peripheral blood using SDS/proteinase K treatment, phenol-chloroform extraction and ethanol precipitation. Genomic DNA of 200 healthy controls was extracted from peripheral lymphocytes.

Genetic analysis

Genotyping of the RAD18 Arg302Gln polymorphism was carried out by polymerase chain reaction using the confronting two-pair primer (PCR-CTPP) technique (Hamajima et al. 2000; Hamajima 2001). According to the sequence of the human *RAD18* gene shown in database, we designed two sets of paired primers. The first set of primers was as follows: forward primer 1, 5′-ATA CCC ATC ACC CAT CTT C-3′ and reverse primer 1, 5′-GTC TTC TCT ATA TTT TCG ATT TCT T-3′ for the A (Gln) allele amplifying a 146 bp band. The second set of primers was as follows: forward primer 2, 5′-TTA ACA GCT GCT GAA ATA GTT CG-3′ and reverse primer 2, 5′-CTG AAA TAG CCC ATT AAC ATA CA-3′ for the G (Arg) allele amplifying a 106 bp band. A 206 bp band was designed between the forward primer 1 and the reverse primer 2. Genomic DNA (20 ng) was assessed in 20 μl of

reaction mixture containing 40 μM of each dNTP, 1X PCR buffer, 8 pmol of the forward primer 1 and reverse primer 2, 24 pmol of the forward primer 2 and reverse primer 1 and 0.5 unit of the Taq DNA polymerase (Takara, Kyoto, Japan). The PCR amplification was initiated by a denaturing step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 64°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min. For genotyping, the PCR products were subjected to electrophoresis in 3% agarose gel with ethidium bromide staining and then visualized on a UV transilluminator. The allele types were determined as follows; 205 and 106 bp for the G/G (Arg/Arg) genotype, 205 and 146 bp for the A/A (Gln/Gln) genotype and 205, 146 and 106 bp for the G/A (Arg/Gln) genotype. In order to confirm the allele types, some PCR products were processed with the Big Dye terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), then analyzed and confirmed on an ABI 3100 sequencer (Applied Biosystems).

Statistical analysis

We compared the allele frequencies of the polymorphism in the *RAD18* gene between NSCLC patient group and healthy control group. The distribution of the *RAD18* genotype (Arg/Arg, Arg/Gln, Gln/Gln) in all of the patients and the controls was tested for adherence to the Hardy–Weinberg equilibrium. The Chi-square test was used to compare the genotype distribution between patients and controls. The odds ratio (OR) and 95% confidence interval (95% CI) were used to estimate the risk of association with genotype. The OR and 95% CI was adjusted for age, gender and smoking habit by an unconditional logistic regression model using the SPSS software Ver.12.0 (SPSS Inc., Tokyo, Japan).

Results

Assessment of cancer risk by RAD18 genotyping

The characteristics of the 159 NSCLC patients and the 200 healthy controls are shown in Table 1. There were no significant differences in gender, age or smoking status between these two groups. Pack-year equivalents were used for smoking status (however, we could not obtain the smoking status for 5 of 159 NSCLC patients).

The representative PCR-CTPP patterns and sequence patterns were shown in Fig. 1a, b, respectively. Significant differences in the genotype frequency were evident between NSCLC patients and controls (Table 2). The frequencies of Arg/Arg, Arg/Gln and Gln/Gln genotype were found to be 29.6, 49.7 and 20.7% in the NSCLC patients and 43.0, 45.5 and 11.5% in the controls, respectively. All of the results fitted the Hardy–Weinberg equilibrium. In comparison to Arg/Arg genotype, the most significantly increased risk was found in NSCLC patients with Gln/Gln genotype with an adjusted OR of 2.57 (95% CI, 1.35–4.89). Thus, this result suggested that the homozygous Gln/Gln genotype has an increased risk of NSCLC.

Table 1 Characteristics of NSCLC patients and healthy controls

	Patients	Controls	*P*-value
	n (%) (*n* = 159)	*n* (%) (*n* = 200)	
Gender			0.874[b]
Male	107 (67.3)	133 (66.5)	
Female	52 (32.7)	67 (33.5)	
Age (years ± SD)[a]	66.2 ± 9.94	65.6 ± 9.42	
Smoking habit			0.909[c]
No-smoker	50 (31.4)	63 (31.5)	
Smoker	104 (65.4)	137 (68.5)	
<20 pack-years	5 (4.8)	17 (12.4)	
≥20 pack-years	97 (92.3)	87 (63.5)	
Unknown	2 (2.9)	33 (24.1)	
Unknown	5 (3.2)	0 (0.0)	

[a] Age shows the mean age of each group with standard deviation

[b] *P*-values were for the differences in the number of males and females between patients and controls and were calculated by Chi-square test

[c] *P*-values were for the differences in the number of smokers and non-smokers between patients and controls and were calculated by Chi-square test

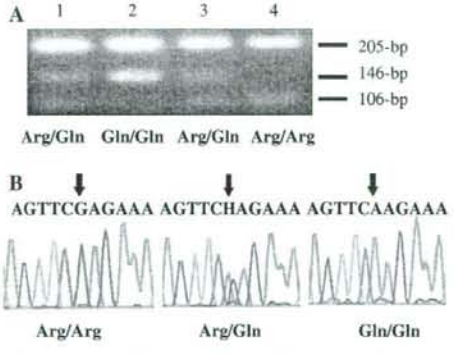

Fig. 1 The single nucleotide polymorphism at codon 302 of the *RAD18* gene. **a** The PCR-CTPP patterns of the *RAD18* SNP. The PCR product was electrophoresed in 3% agarose gel. Two fragments of 205 and 106 bp show the G/G (Arg/Arg) genotype, two fragments of 205 and 146 bp show the A/A (Gln/Gln) genotype, and three fragments of 205-, 146 and 106 bp show the G/A (Arg/Gln) genotype. The case number and genotypes are shown at the *top* and *bottom*, respectively. **b** The direct sequence patterns of the *RAD18* SNP. The SNP, Arg (CGA) or Gln (CAA), is indicated by an *arrow* above the sequence

Table 2 The *RAD18* genotypes in patients and controls

RAD18	Patients	Controls	*P*-value	OR (95% CI)	
Genotype	*N* (%)	*N* (%)		Crude	Adjusted[b]
Arg/Arg	47 (29.6)	86 (43.0)		1 (Reference)	1 (Reference)
Arg/Gln	79 (49.7)	91 (45.5)	0.051[a]	1.59 (1.00–2.53)	1.60 (1.00–2.56)
Gln/Gln	33 (20.7)	23 (11.5)	0.003[a]	2.63 (1.38–4.98)	2.57 (1.35–4.89)
Total	159	200			
Allele frequencies			0.002		
Arg	173 (54.4)	263 (67.8)			
Gln	145 (45.6)	137 (34.2)			

[a] *P*-values were calculated for the difference in genotype frequencies against Arg/Arg by Chi-square test

[b] ORs were adjusted for age, gender and smoking status. Patients whose smoking status was not known were excluded when ORs were calculated

The association between the *RAD18* genotype and clinicopathological features

We next analyzed the relationship between the genotype distribution and the clinicopathological parameters. Strong association between the risk of lung squamous-cell carcinoma (LSC) and genotype distribution was shown in Table 3. The adjusted OR of LSC patients with Gln/Gln genotype was 4.40 (95% CI, 1.60–12.1), whereas the same genotype exhibited a marginal risk for lung adenocarcinoma (LAD) with a borderline significance (adjusted OR = 1.97, 95% CI, 0.94–4.12). Differentiated grade, TNM classification, gender and smoking habit were not associated with the frequency of genotype or allele (Table 4).

Discussion

In the present study, we examined whether the SNP at codon 302 in the *RAD18* gene is associated with the risk for development of NSCLC, and found significant differences in the genotype distribution between the NSCLC patients and the healthy controls. Our findings suggest that this SNP is associated with the development of the NSCLC, and the susceptibility to the NSCLC is enhanced by the Gln/Gln genotype. However, this SNP does not appear to be associated with progression or metastasis of the NSCLC, as the *RAD18* genotype showed no correlation with the clinicopathological characteristics, except histological types. The Gln/Gln genotype was detected more frequently in the NSCLC patients, and the individuals with the Gln/Gln genotype showed a 2.6-fold higher risk of NSCLC. Furthermore, as for the LSC patients, a strong association between the Gln/Gln genotype and the development risk was detected (OR = 4.40, 95% CI = 1.60–12.1). Notably, the heterozygotes (Arg/Gln) exhibited an intermediate risk, still with statistic significance, for both whole NSCLC (OR = 1.60, 95% CI = 1.00–2.56) and LSC (OR = 2.40, 95% CI = 1.09–5.29), indicating a dose-response effect of the Gln allele. This shows that the Gln allele may be defined as the responsive risk-allele. It would be of great interest to see the effects of the SNP on incidence of NSCLC in Europeans and Africans, since the frequency of the individuals with the Gln/Gln genotype is much higher (60%) in these races than in Asian people (8–18%)(rs#373572 in the dbSNP). Giving the high risk of the Gln/Gln genotype for LSC among NSCLC, the ethnic difference may well explain, at least in part, the higher proportion of LSC among NSCLC in Caucasians than in Asians.

Table 3 Association between the RAD18 genotype distribution and histological cell type of patients

Characteristics	Genotype (%)				OR[a] (95% CI)	
	Arg/Arg	*Arg/Gln*	*Gln/Gln*	Total	*Arg/Gln*	*Gln/Gln*
Controls	86 (43.0)	91 (45.5)	23 (11.5)	200		
All patients	47 (29.6)	79 (49.7)	33 (20.7)	159	1.60 (1.00–2.56)	2.57 (1.35–4.89)
LAD	34 (32.4)	53 (50.5)	18 (17.1)	105	1.51 (0.89–2.56)	1.97 (0.94–4.12)
LSC	11 (22.9)	25 (52.1)	12 (25.0)	48	2.40 (1.09–5.29)	4.40 (1.60–12.1)
Others	2 (33.3)	1 (16.7)	3(50.0)	6		

LAD lung adenocarcinoma, *LSC* lung squamous-cell carcinoma

[a] ORs were adjusted for age, gender and smoking status. The Arg/Arg genotype of healthy controls was defined as the reference

Table 4 Association between the *RAD18* genotype and clinicopathological parameters of patients

Characteristics	Genotype (%)					Allele (%)		
	Arg/Arg	*Arg/Gln*	*Gln/Gln*	Total	*p*-value	*Arg*	*Gln*	*P*-value
Differentiated grade								
Well	18 (34.6)	23 (44.2)	11 (21.2)	52		59 (56.7)	45 (43.3)	
Moderate	16 (25.8)	35 (56.5)	11 (17.7)	62	0.420[a]	67 (54.0)	57 (46.0)	0.683[a]
Poor	9(25.0)	19(52.8)	8(22.2)	36	0.613[a]	37 (51.4)	35 (48.6)	0.484[a]
Unknown	3	3	3	9				
TNM classification								
I	31 (33.0)	43 (45.7)	20 (21.3)	94		105 (55.9)	83 (44.1)	
II, III, IV	16 (26.7)	33 (55.0)	11 (18.3)	60	0.530	65 (54.2)	55 (45.8)	0.772
Unknown	0	3	2	5				
Gender								
Male	28 (26.2)	57 (53.3)	22 (20.5)	107		113 (52.8)	101 (47.2)	
Female	19 (36.5)	22 (42.3)	11 (21.2)	52	0.254	60 (57.7)	44 (42.3)	0.133
Unknown	0	0	0	0				
Smoking habit								
Smoker	25 (25.3)	53 (53.5)	21 (21.2)	99		103 (52.0)	95 (48.0)	
No-smoker	22 (40.0)	22 (40.0)	11 (20.0)	55	0.558	66 (60.0)	44 (40.0)	0.351
Unknown	0	4	1	5				

[a] *P*-values were calculated against Well-differentiated grade by Chi-square test

We recognize that this specific population of cancer patients does not seriously deviate from the general Japanese population because Japan is an almost racially homogeneous nation and Okayama has experienced population influxes from other areas, such as Tokyo and Osaka (the urban city representing Japan) and the Chugoku and Shikoku Districts (surrounding Okayama).

RAD18 is one of the most important proteins involved in the PRR pathway. In the PRR pathway, an interaction between RAD18 and RAD6 is essential for carrying out PRR (Wood et al. 2003; Bailly et al. 1994; Dohmen et al. 1991; Sung et al. 1991b). Since RAD6, which has no DNA binding activity, interacts with RAD18, it has been proposed that RAD18 recruits RAD6 to the site of DNA damage via its physical interaction where RAD6 and its complex then modulate stalled DNA replication through their ubiquitin-conjugating activity (Haracska et al. 2004; Watanabe et al. 2004). There have been reports that the proliferating cell nuclear antigen (PCNA), a DNA polymerase sliding clamp that is involved in DNA synthesis and repair, is a substrate of the ubiquitin conjugating enzyme, and it is ubiquitinated in a RAD18- and RAD6-dependent manner (Hoege et al. 2002; Stelter and Ulrich 2003; Kannouche et al. 2004). Therefore, the monoubiquitination of PCNA through RAD18 and RAD6 is necessary for carrying out DNA PRR. RAD18 interacts with RAD6 through the RAD6-binding domain in the C-terminal region (AA371-410) (Fig. 2). Considering that Gln/Gln genotype was detected more frequently in NSCLC patients, substitution of Arg by Gln may reduce the RAD6-binding activity. Furthermore, RAD18 has several other functional domains as well, such as the RING-finger motif (Costa et al. 2006), zinc-finger motif (Mackay and Crossley 1998; Akhtar and Becker 2001) and E3 ubiquitin-ligase domain (Marchler-Bauer et al. 2005). The RING-finger motif, residing in the N-terminal region, and the E3 ubiquitin-ligase domain together confer an ubiquitin ligase activity on RAD18. The RAD18 Arg302Gln polymorphism is located in the E3 ubiquitin-ligase domain (Fig. 2). Therefore, this SNP may affect the E3 ubiquitin-ligase activity of RAD18. It is also possible that this SNP may affect the interaction between RAD18 and other proteins involved in PRR through its structural change, which is generated by the substitution of one amino acid residue, a basic amino acid residue (Arg) to a neutral residue (Gln).

Our data provide evidence for an association between the RAD18 Arg302Gln polymorphism and the risk of NSCLC. It is possible that this polymorphism may influence susceptibility to a variety of human cancers through incomplete PRR. The sample size we analyzed was small; however, we recognized that our findings were true because the findings of this study are statistically significant. Analysis with threefold or more of normal control population against our patient population will define more precise values for statistical analysis. Further study with sufficiently larger populations and functional analysis of

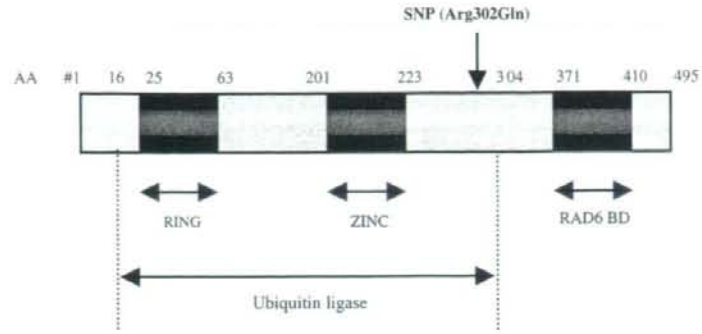

Fig. 2 The location of the polymorphism and the functional motifs of RAD18 protein. The SNP (Arg302Gln) is indicated by an *arrowhead* above the motif. The motifs of the RAD18 protein are depicted in *dark gray* and/or by *arrows*. RING, RING-finger motif (AA25-63); ZINC, zinc-finger motif (AA201-223); Ubiquitin ligase, E3 ubiquitin ligase domain (AA16-304); RAD6 BD, RAD6 binding domain (AA371-410). AA #, amino acid number

this polymorphism will be required in order to clarify this issue.

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Long-lasting alterations of the immune system by ionizing radiation exposure: Implications for disease development among atomic bomb survivors

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Abstract

Purpose: The immune systems of the atomic-bomb (A-bomb) survivors were damaged proportionately to irradiation levels at the time of the bombing over 60 years ago. Although the survivor's immune system repaired and regenerated as the hematopoietic system has recovered, significant residual injury persists, as manifested by abnormalities in lymphoid cell composition and function. This review summarizes the long-lasting alterations in immunological functions associated with atomic-bomb irradiation, and discusses the likelihood that damaging effects of radiation on the immune system may be involved partly in disease development so frequently observed in A-bomb survivors.

Conclusions: Significant immunological alterations noted include: (i) attrition of T-cell functions, as reductions in mitogen-dependent proliferation and interleukin-2 (IL-2) production; (ii) decrease in helper T-cell populations; and (iii) increase in blood inflammatory cytokine levels. These findings suggest that A-bomb radiation exposure perturbed one or more of the primary processes responsible for T-cell homeostasis and the balance between cell renewal and survival and cell death among naïve and memory T cells. Such perturbed T-cell homeostasis may result in acceleration of immunological aging. Persistent inflammation, linked in some way to the perturbation of T-cell homeostasis, is key in addressing whether such noted immunological changes observed in A-bomb survivors are in fact associated with disease development.

Keywords: *Immunology, inflammation, atom bomb effects, cytokines, flow cytometry, epidemiology*

Introduction

More than 60 years after the atomic bombings of Hiroshima and Nagasaki, there are still significant uncertainties as to how and to what extent atomic-bomb (A-bomb) irradiation has affected the health of individuals, and their susceptibilities to different diseases. While epidemiological studies have helped to identify various exposure-disease relationships, additional studies on underlying mechanisms are needed to fully understand the biological bases of such relationships.

Many human diseases appear to be the consequence of abnormalities of the immune system. As such, in order to gain further insight into mechanisms of radiation-induced diseases, it might be useful to study the origin of these radiation-associated disorders from an immunological point of view. Exposure to radiation is thought to affect host immune surveillance, but little is known about the direct relationship between radiation effect on the immune system and some of the most significant, late-arising, radiation-induced diseases.

The immune system of the A-bomb survivors was damaged proportionately to the intensity of the A-bomb ionizing irradiation, and as a result of induced cytotoxicity and excessive cell loss. Due to the robustness of the cell repopulation processes, the damaged hematopoietic system of survivors had largely and most surely recovered within a few months following the A-bomb radiation exposures (Oughtersen & Warren 1956, Ohkita 1975). However, even 60 years after radiation exposure, lymphocyte and hematopoietic stem cell populations still bear residual molecular lesions, e.g., somatic mutations and chromosome aberrations, associated with

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prior exposures. (Hakoda et al. 1988, Langlois et al. 1987, Kyoizumi et al. 1989, Awa 1991). Further, there is accumulating evidence of persistent radiation effects on lymphoid tissues, specifically in terms of cell composition and function (Akiyama et al. 1983, Kusunoki et al. 1988, Akiyama et al. 1989, Fujiwara et al. 1994, Kusunoki et al. 1998, Kusunoki et al. 2001, Kusunoki et al. 2002a, Kusunoki et al. 2003, Yamaoka et al. 2004) (Figure 1).

An earlier review by Akiyama (Akiyama 1995) summarized radiation-associated changes in various immunological parameters and listed a number of diseases possibly related to dysfunctional immune systems of the A-bomb survivors. This report attempts to extend the information presented in this earlier review by providing new insights into immunological mechanisms underlying radiation-related diseases based on more current information.

We have recently obtained evidence that supports the possible involvement of immunological alterations in select types of late-arising diseases in A-bomb survivors. Accordingly, this manuscript summarizes data on how A-bomb radiation exposure may have caused long-lasting alterations in immunological functions, and how the damaging effects of A-bomb radiation on the immune system may be linked to specific late-arising disease.

Lymphocyte population alterations observed in A-bomb survivors

Peripheral blood lymphocytes are composed largely of various types of functionally mature cells, and arise from common hematopoietic stem cells. It is uncertain whether or not hematopoietic stem cells of A-bomb survivors possess defects that affect production of any particular type of descendant cells, even though it is clear that these stem cells have genetic lesions such as chromosome aberrations and somatic gene mutations. Indeed, we have isolated both functionally and phenotypically heterogeneous mature lymphocyte populations from A-bomb survivors, apparently indicating that they were derived from genetically aberrant stem cells (Hakoda et al. 1989, Kusunoki et al. 1995, Nakano et al. 2004). The ability of peripheral blood T cells to proliferate *in vitro*, in the presence of sufficient exogenous growth stimuli, did not appear to be affected by A-bomb radiation (Kusunoki et al. 2001). Therefore, alterations in the composition of A-bomb survivors' peripheral lymphocyte populations are perhaps more likely due to the effect of radiation on lymphocyte differentiation, proliferation and/or cell death.

Lymphocyte subpopulations differ in size for men and women, and with age. In order to more precisely

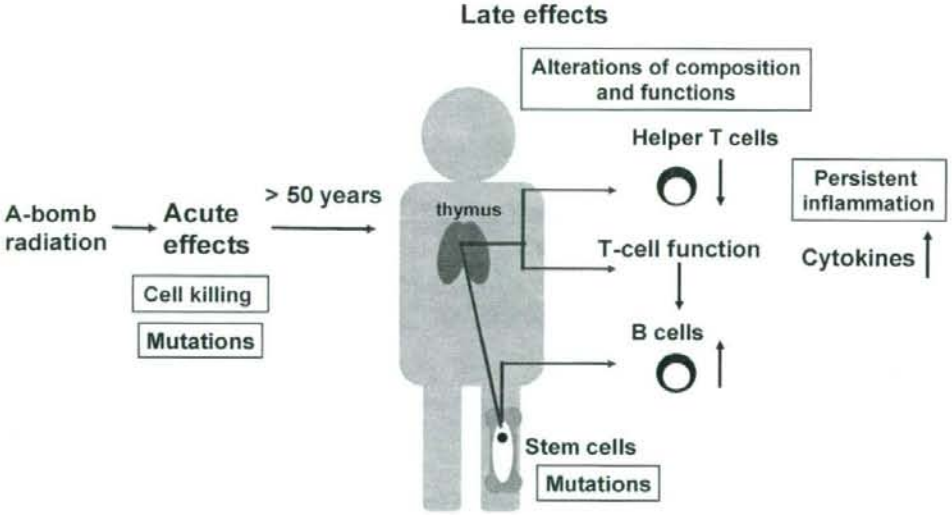


Figure 1. Acute and late effects of A-bomb radiation on the human immune system. The immune system was dose-dependently damaged in A-bomb survivors, mainly due to radiation-induced cell death. Several months after radiation exposure, the system regenerated as the hematopoietic system had nearly recovered from the damage in the survivors. However, there still remain lymphocyte and hematopoietic stem cell populations that bear radiation-induced DNA damage, such as somatic mutations and chromosome aberrations, even more than 50 years after radiation exposure. In addition, we can still observe significant effects of the previous radiation exposure on lymphoid cell composition and function in the immune system of the survivors, that is, a decrease of CD4 helper T-cell population in association with attenuated T-cell function and an increase of B-cell population. Such radiation-induced alterations in the immune system may lead to persistent inflammation among A-bomb survivors. In support of this hypothesis, we have observed radiation-dose-dependent increases in the levels of inflammatory cytokines.

understand how A-bomb radiation has reduced or increased the proportion of lymphocyte subpopulations, effects of gender and age are necessarily included in analyses. Effects of gender, age, and A-bomb radiation dose on major lymphocyte subpopulations in subgroups of A-bomb survivors are listed in Table I.

(1) Cluster of differentiation (CD)- 4 and CD8 T-cell populations

Peripheral blood T cells are composed primarily of CD4 and CD8 T cells that recognize antigens presented with major histocompatibility complex (MHC) class-II and class-I molecules, respectively. The main effector functions of CD4 T cells are to activate macrophages in cell-mediated immune responses, and to promote B cell antibody production in humoral immune responses; the effector functions of CD8 T cells are to recognize and kill host cells infected with viruses or other intracellular microbes. The proportion of CD4 T cells in peripheral blood lymphocyte populations was found to be significantly lower in males than females, and to decrease with age (Table I). The proportion of CD8 T cells showed a similar gender difference, but did not change with aging. Statistical analysis using gender- and age-adjusted values for individuals revealed a radiation-dose dependent decrease in the proportion of CD4 T cells. A similar effect of radiation on CD4 T-cell populations has been reported in different subgroups of Hiroshima survivors (Kusunoki et al. 1998, Kusunoki et al. 2002a, Kusunoki et al. 2003, Yamaoka et al. 2004). On the other hand, no significant radiation effect has been observed in the proportion of the CD8 T-cell population (Kusunoki et al. 1998, Kusunoki et al. 2002a, Kusunoki et al. 2003, Yamaoka et al. 2004). These findings suggest a possible long-lasting effect of radiation that could be important in the immunology of disease development in A-bomb survivors.

(2) Naïve and memory T-cell populations

It is widely accepted that the peripheral T-cell system comprises two distinct T-cell populations: (i) naïve T cells – which have not encountered antigen exposure since their maturation, and (ii) memory T cells – which mediate rapid and enhanced (i.e., memory) responses to second and subsequent exposure to antigens (Goldrath & Bevan 1999). These populations seem to contribute differently to immunological defense against infections, each with a different and diverse repertoire of antigen recognition machineries (Goldrath & Bevan 1999). Among our study populations, it is apparent that the proportion of naïve T cells declines with age and with radiation dose, and that these trends are significant for both CD4 and CD8 T-cells (Table I). A decrease in the number of naïve T-cell populations has also been

Table I. How A-bomb radiation altered composition of lymphocyte subsets.

Lymphocyte subsets	Effects			Study period and number of study subjects[a] (*N*)	Reference
	Gender	Age (10 years)	Radiation (Gy)		
T cells					
CD4 Total	F > M (5%)*	Decrease (5%)*	Decrease (2%)*	1992–1995 (723)	Kusunoki et al. 2002a
Naïve					
$CD45RA^+$	F > M (3%)*	Decrease (8%)*	Decrease (5%)*	1992–1995 (723)	Kusunoki et al. 2002a
$CD45RO^-/CD62L^+$	NS[b]	Decrease (25%)*	Decrease (9%)*	2000–2003 (533)	Yamaoka et al. 2004
Memory					
$CD45RA^-$	F > M (8%)*	NS	NS	1992–1995 (723)	Kusunoki et al. 2002a
$CD45RO^+/CD62L^+$	F > M (10%)*	Decrease (11%)*	NS	2000–2003 (533)	Yamaoka et al. 2004
$CD45RO^+/CD62L^-$	F > M (7%)*	Increase (8%)*	NS	2000–2003 (533)	Yamaoka et al. 2004
CD8 Total	NS	NS	NS	1992–1995 (723)	Kusunoki et al. 2002a
Naïve					
$CD45RO^-/CD62L^+$	F > M (19%)*	Decrease (35%)*	Decrease (8%)*	2000–2003 (533)	Yamaoka et al. 2004
Memory					
$CD45RO^+/CD62L^+$	NS	NS	Increase (12%)*	2000–2003 (533)	Yamaoka et al. 2004
$CD45RO^+/CD62L^-$	M > F (12%)*	Increase (6%)^{mg}	Increase (8%)*	2000–2003 (533)	Yamaoka et al. 2004
B cells	F > M (5%)*	Decrease (7%)*	Increase (8%)*	1988–1992 (411)	Kusunoki et al. 1998
NK cells	M > F (20%)*	Increase (21%)*	NS	1988–1992 (411)	Kusunoki et al. 1998

[a]Study subjects were selected from participants in the Adult Health Study (Kodama et al. 1996b) of the Radiation Effects Research Foundation (RERF) in Hiroshima, distributed almost equally by age, gender, and dose. [b]Not significant ($p > 0.1$). Associations of percentage of each lymphocyte subpopulation (*percentage*) with age at the time of examination (*age*), gender, and the radiation dose (*dose*) were analyzed based on a following multiple regression model (Kusunoki et al. 2001), assuming that the percentage of each lymphocyte cell subpopulation related to each explanatory variable in a logarithmic manner, $\log(percentage) = \alpha + \beta_1 age + \beta_2 gender + \beta_3 dose$, where gender = 0 for male and = 1 for female. The numbers in parentheses denote % changes between gender, per 10 years, or per Gy; $^*p < 0.05$, $^{mg}p < 0.1$.

observed in other studies, such as radiotherapy patients (Watanabe et al. 1997). A plausible mechanism for this radiation-induced effect is that the naïve T-cell pools are depleted as a result of an insufficient input of new T cells from the thymus from which the majority of the naïve T cells develop. Reduced production of naïve T cells may compromise the host's ability to mount an effective immune response to microbial challenge not previously experienced by the host.

While the percentages of memory CD4 T cells did not significantly change with radiation exposure, the percentages of memory CD8 T cells in A-bomb survivors did increase, and significantly so with radiation dose (Yamaoka et al. 2004). This change was not simply the result of a clonally driven expansion of $CD28^-$ and $CD57^+$ CD8 T-cell populations that occur frequently in older individuals (Yamaoka et al. 2004). Although the basis for this differential radiation effect in memory CD4 and CD8 T-cell populations is uncertain, it is likely due to different regulatory processes by which memory CD4 and CD8 T-cell pools maintain their size (Mackall et al. 1997).

(3) Other lymphocyte populations

B cells represent the second major class of lymphocytes that comprise the adaptive immune arm of the host. Plasma cells that differentiate from B cells produce antibodies to protect against infections by microbes and to eliminate extracellular pathogens, usually in response to antigenic stimuli, and they do this with the help of T cells. In a manner similar to T cells, the proportion of B cells in peripheral blood lymphocyte population significantly decreased with age, and was higher in females than in males (Table I). However, in contrast to the effects of radiation on CD4 and naïve T-cell populations, the proportion of B cells in the peripheral blood lymphocyte fraction increased as the intensity of radiation exposure increased (Kusunoki et al. 1998).

Unlike T and B cells, the number of $CD3^-$ $CD16^+CD56^+$ natural killer (NK) cells that mediate innate immune responses to some types of viruses and cancers increase with age and was higher among males than females (Table I). However, no significant effect from A-bomb radiation on the proportion of NK cells has been observed (Kusunoki et al. 1998).

Recent studies have indicated that $CD4^+CD25^+$ regulatory T cells play crucial roles in suppression of host immune responses, especially the responses to self antigens (von Herrath & Harrison 2003). NK T cells that share properties of both NK and T cells and that are defined by the expression of a peculiar T-cell receptor (TCR) Vα chain encoded in humans by the homologue invariant *Vα24-JαQ* gene rearrangement have also been suggested to play a pivotal role in the interplay between innate and acquired immune responses by directing the polarization of T-cell function toward T-helper type 1 (Th1) or type 2 (Th2) pathways (Taniguchi & Nakayama 2000). The lymphocytes fraction exhibiting $CD3^+CD16^+$ $CD56^+$ phenotype contains NK T cells, and this fraction was found to increase in the blood of individuals who participated in cleanup activities for the Chernobyl accident (Kuzmenok et al. 2003). However, our previous examination regarding the $CD3^+$ $CD56^+$ T cell population (which also contains NK T cells) in A-bomb survivors did not reveal any significant association with radiation dose (Kusunoki et al. 1998). It remains to be seen whether radiation exposure affects these important lymphocyte subsets.

Lymphocyte function alterations observed in A-bomb survivors

Table II lists effects of A-bomb radiation on lymphocyte functions that we have observed among subgroups of A-bomb survivors.

(1) Cell-mediated immunity

There are dose-dependent decreases in T-cell responses to mitogens, such as phytohemagglutinin (PHA), alloantigens (mixed lymphocyte reaction,

Table II. Radiation-related alterations in cellular immune functions among A-bomb survivors.

Cell type	Function	Radiation-related alteration[a]	Study period and number of study subjects[b] (*N*)	Reference
T cells	PHA response	Decrease	1974–1977 (683)	Akiyama et al. 1983
	MLR	Decrease	1984–1985 (139)	Akiyama et al. 1989
	IL-2 production	Decrease	1988–1992 (410)	Kusunoki et al. 2001
	SAg response	Decrease	1992–1995 (723)	Kusunoki et al. 2002a
NK cells	K562 cell lysis	NS	1983–1986 (1316)	Bloom et al. 1988

[a]Radiation-related alterations were analyzed using a standard multiple regression method with adjusting for gender and age. [b]Study subjects were selected from participants in the Adult Health Study in Hiroshima, distributed almost equally by age, gender, and dose. PHA, phytohemagglutinin; MLR, mixed lymphocyte reaction; IL-2, interleukin-2; SAg, superantigen; Ab, antibody; NK, natural killer; NS, not significant.

MLR), and superantigen (SAg) staphylococcal enterotoxin, in A-bomb survivors (Table II). These functional alterations are consistent with our observations of compositional shifts of T lymphocytes of A-bomb survivors (Table I), e.g., the decrease in the proportion of CD4 helper T-cell population. The T-cell proliferative responses to SAgs positively correlated with the CD45RA-positive (naïve) CD4 T-cell percentages, but not with the CD45RA-negative (memory) CD4 T-cell percentages (Kusunoki et al. 2002a). The radiation dose-dependent reductions in T-cell responses to mitogenic stimuli that are observed in A-bomb survivors are likely to be associated with a decrease in the proportion of naïve CD4 T cells, i.e., the observed alterations of T-cell functions may be due to reduced numbers of T cells resulting from a radiation exposure-induced insufficiency in generating new T cells (Kusunoki et al. 2002a). Increased losses of naïve CD4 T cells following their transit into memory CD4 T-cell pools, and/or as a consequence of radiation-induced apoptosis may also be responsible in part of reduced numbers of T cells. A study using limiting dilution analysis revealed an A-bomb radiation-dose-dependent decrease in the percentages of T cells capable of producing interleukin-2 (IL-2) (Kusunoki et al. 2001). By contrast, similar limiting dilution analyses did not show significant dose-response relationships for T cells and their proliferating ability in response to exogenous mitogenic stimuli, including recombinant IL-2 (Kusunoki et al. 2001). In this study, we first assumed that CD4 T cells were the cells primarily responsible for producing IL-2, and then estimated how many cells in the CD4 T-cell population under test were actually producing IL-2. The results indicated that CD4 T-cell populations of the survivors contained significantly fewer IL-2 producing cells than those of controls, suggesting that the decreases in the IL-2-producing cell fractions we have observed in A-bomb survivors may not be entirely a function of decreases in CD4 T-cell numbers, and may be partly due to deficits in IL-2 production among the CD4 T-cell populations of individuals. It may therefore be that IL-2 production *per se* has in fact been reduced by A-bomb radiation exposure.

(2) Humoral immunity

Earlier studies in the 1970s did not detect any significant radiation effects on the levels of circulating immunoglobulins (Ig) in A-bomb survivors (reviewed in Akiyama 1995). However, a large-scale study (Fujiwara et al. 1994) revealed radiation-dose-dependent increases in IgM (in both males and females) and IgA (in females) levels. Another study has demonstrated in a subset of Hiroshima survivors that IgM, IgG and IgA levels tend to increase with radiation dose (Hayashi et al. 2005). The positive results from these two recent studies may be largely attributable to improvements in assay systems that provide for more sensitive measurements. The reason for this enhanced B-cell immune response in the survivors is unclear: It may be that an increased inflammatory reaction, due to a deficit of helper T cells, is involved in the enhanced B-cell responses of survivors. Recently we found that there was a positive association between C-reactive protein (inflammation marker) and anti-*Chlamydia pneumoniae* antibody levels especially in more heavily exposed ($\geq$1 Gy) A-bomb survivors, although the antibody levels appeared to decrease with radiation dose among a total survivor population examined (Hakoda et al. 2006). This suggested that the diminished immune response to *Chlamydia pneumoniae* might be related to chronic inflammatory reactions, which is likely a reflection of an active state of infection in those survivors exposed to relatively high doses. Alternatively, elevated Th2 cytokines levels such as IL-6 levels (Hayashi et al. 2003b) may be associated with enhanced antibody production in A-bomb survivors. However, it is unlikely that A-bomb irradiation has shifted the balance of regulatory mechanisms in favor of Th2 immunity (see below, subsection 3) Th1/Th2 balance).

Prevalence of hepatitis B virus (HBV) carriers appeared to be increased among A-bomb survivors (Kato et al. 1983, Neriishi et al. 1995). This observation has fully been confirmed in a recent study (Fujiwara et al. 2003) where the seropositive rate of HBV surface antigen (HBsAg) has been analyzed, along with information about blood transfusions, family history of liver disease, and HBV antibody status. Interestingly, the proportion of HBsAg-positive persons among those positive for either HBsAg, or surface or core hepatitis B antibody was found to be significantly increased in the more heavily exposed individuals, especially in those individuals who had received blood transfusions (Fujiwara et al. 2003). This result suggested that the prior A-bomb irradiation might have negatively affected the ability of the individuals' immune system to eliminate HBV infection, possibly acquired by transfusion. By contrast, hepatitis C virus (HCV) infections were not influenced by the extent of irradiation, as reflected by the exposure-independent prevalence of anti-HCV, anti-HCV titers among A-bomb survivors (Fujiwara et al. 2000). Although cell-mediated immunity is thought to play a critical role in the clearance and control of hepatitis virus, whether any alterations of lymphocyte populations and functions are involved in the anti-hepatitis virus antibody response, or in virus-mediated liver pathogenesis, have not yet been addressed among A-bomb survivors.

It has been reported that there was increased prevalence of high titered antibody responses to the early antigen of Epstein-Barr virus (EBV) among A-bomb survivors, suggesting a possible frequent reoccurrence and reactivation of EBV (Akiyama et al. 1993). However, no radiation-dose-dependent alterations were noted in the prevalence of antibody responses to either EBV capsids or EBV nuclear antigens (Akiyama et al. 1993), human T lymphotropic virus type I (Matsuo et al. 1995), or cytomegalovirus (Hakoda et al. 2006). The frequency of finding antibody to *Chlamydia pneumoniae*, but not to *Helicobacter pylori*, appeared to decrease with radiation dose among A-bomb survivors (Hakoda et al. 2006). Viral and bacteria infections in infants generally contracted prior to irradiation are thought to persist throughout life, and to occasionally induce chronic inflammations and cancers. Associations between humoral immune responses to these microbial infections and diseases risks among A-bomb survivors remain ill-defined and need to be more thoroughly investigated.

(3) Th1/Th2 balance

From the viewpoint of the Th1/Th2 paradigm – and based on the observations mentioned above – we have hypothesized that A-bomb irradiation triggered decreases in cellular immune responses controlled by Th1 cells while augmenting humoral immune responses controlled by Th2 cells (Kusunoki et al. 2001). This hypothesis was investigated by measuring levels of plasma cytokines related to Th1- or Th2-dominant status and by enumerating the numbers of Th1 and Th2 cells in the peripheral blood using cell surface markers for chemokine receptor, CXCR3 and prostaglandin D receptor, CRTH2, respectively (Cosmi et al. 2000). Results obtained indicated radiation-dose dependent elevations of cytokine levels for both the Th2-related cytokine, IL-6, and for the Th1-related cytokines, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Hayashi et al. 2005). These results indicated that the A-bomb survivors had enhanced production of inflammatory cytokines, but not a Th1/Th2 imbalance. No significant effect of A-bomb irradiation has been found on the ratio between Th1 and Th2 cells (unpublished observation). Even though A-bomb survivors appeared to have T cells that have a diminished ability to produce IL-2 (Kusunoki et al. 2001), it is unlikely that the A-bomb irradiation has significantly shifted the host T-cell immunity in favor of either Th1 or Th2 cells.

(4) Innate immunity

The innate immune system is composed of a variety of distinct cellular and non-cellular components, including; epithelial cell barriers; phagocytic cells such as neutrophils, dendritic cells, and macrophages; NK cells; the blood complement system; and cytokines, primarily made by mononuclear phagocytes. Earlier studies on functions of blood phagocytic cells did not show any radiation effects among the A-bomb survivors (reviewed in Akiyama 1995). Similar to the observations on the concentration of blood NK cells in A-bomb survivors, we could not detect any significant radiation effect on NK cell activity when tested for cell-mediated cytotoxicity against K562 target cells *in vitro* (Bloom et al. 1988). A study by Neriishi et al. has shown that blood leukocyte counts significantly increased with radiation dose in A-bomb survivors (Neriishi et al. 2001). Plasma levels of mononuclear phagocyte-associated inflammatory cytokines, IL-6 and TNF-α, were found to be higher in more heavily exposed survivors (Hayashi et al. 2005). The innate immune system is quite responsive to exogenous stresses, e.g., acute infections, mental stress; therefore, the assessed parameters of innate immunity were likely affected by time of examination, and by variable health status of individuals under test. It is recognized currently that innate immunity provides to the host not only a powerful early defense mechanism against infections, but also serves to instruct the adaptive immune system and associated T and B lymphocytes to respond to infectious microbes (Iwasaki & Medzhitov 2004). Unfortunately however, the long-term effects of prior acute irradiation on the innate immune system, and on its interaction with adaptive immunity, have not systematically been investigated.

(5) Autoimmunity

Previously, it was reported that autoimmune hypothyroidism increased in Nagasaki A-bomb survivors who were exposed to approximately 0.7 Gy (Nagataki et al. 1994). However, more recently Imaizumi et al. reported finding no significant dose-response relationship for positive antithyroid autoantibodies, antithyroid antibody-positive hypothyroidism, or Graves' disease in A-bomb survivors from Hiroshima and Nagasaki that had been comprehensively analyzed with advanced screening techniques to diagnose these thyroid diseases (Imaizumi et al. 2006). As already summarized in an earlier review (Akiyama 1995), there has been no clinical or epidemiological evidence that supports the idea that there is an increase of autoimmune disease among A-bomb survivors. Lack of an obvious radiation exposure-related imbalance of the Th1/Th2 immune response is consistent with the lack of finding on significant adverse autoimmunity within A-bomb survivors.

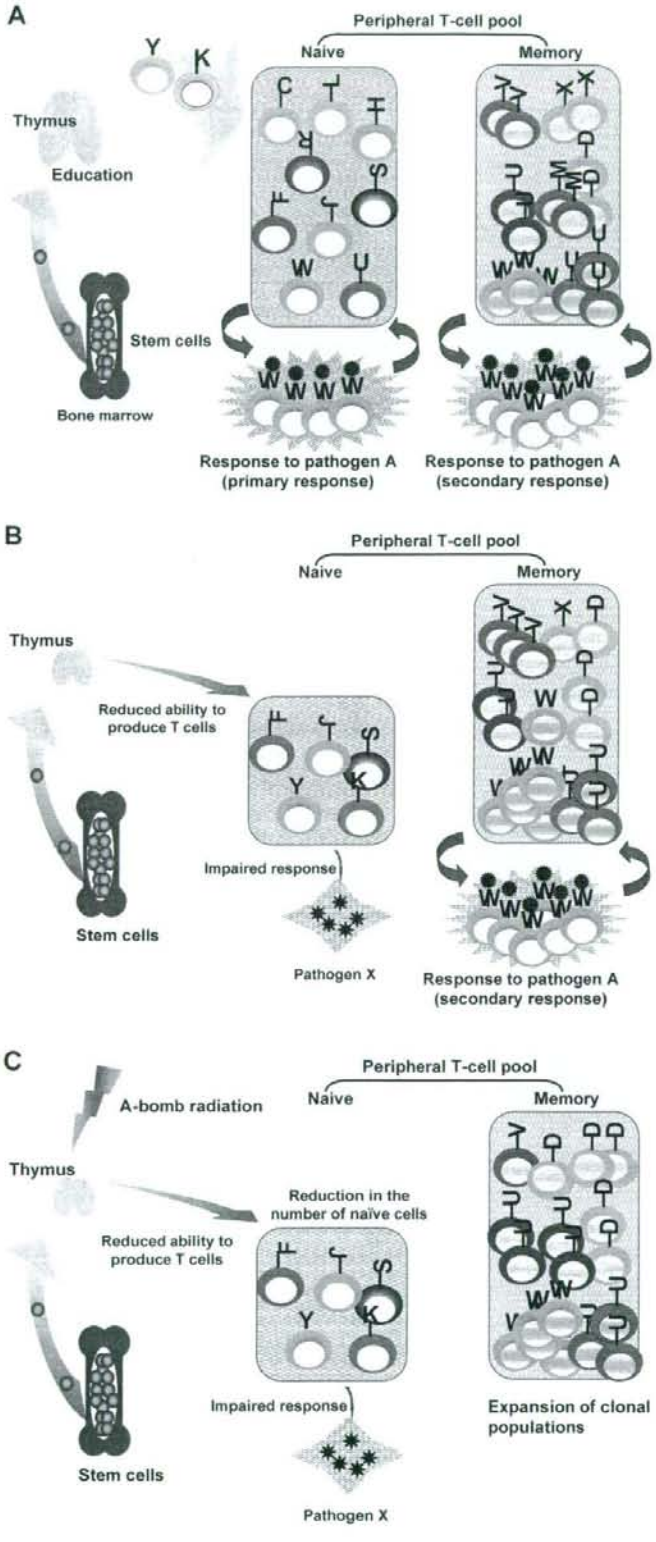
Possible perturbation of T-cell homeostasis in A-bomb survivors

In the T-cell system, a constant supply of phenotypically diverse lymphocyte subsets is maintained, despite the emergence of new lymphocytes and the tremendous expansion of individual clones that occur in response to antigens. This homeostasis in the T-cell system is achieved by the balance between renewal and death among naïve and memory T cells, and by the independent maintenance of the size of these T-cell populations (Goldrath & Bevan 1999) (Figure 2A). Although maintenance of both naïve and memory T-cell pools is essential in protecting the host against invasion by pathogens, the ability to accurately maintain these pools is believed to decline with age (Pawelec & Solana 1997). In the elderly, the naïve T-cell pool decreases due to reduced production of new T cells in the thymus so that responses to antigens are impaired when compared with younger individuals (Miller 1996, Mackall & Gress 1997, Rufer et al. 2001) (Figure 2B). Although the size of the memory T-cell pool remains relatively constant, regardless of age, a fraction of these cells occasionally and preferentially will proliferate, resulting in clonally expanded populations within the memory T-cell pool of older individuals. If such clonally expanded subpopulations appear and constitute a major fraction of the memory T-cell pool, this may result in distortions of the antigen recognition repertoire of memory T-cell population. However, it remains to be determined whether such T-cell alterations lead to the attenuation of immunological memory as related to microbial defense.

It is likely that naïve CD4 and CD8 T-cell pools of A-bomb survivors are not properly maintained, as the numbers of naïve CD4 and CD8 T cells are lower than those in unexposed controls of the same age; this despite more than 50 years after the bombing (Table I) (Yamaoka et al. 2004). This could mean that the naïve T-cell pool was compromised after radiation-induced damage of the T-cell system, and never fully recovered (Figure 2C). In contrast, memory T-cell pools of A-bomb survivors appeared to be almost normal (CD4) or larger (CD8) in size than in controls (Yamaoka et al. 2004). However, we have demonstrated that the extent of the deviation of T-cell receptor repertoire of memory CD4 T cells significantly increased with radiation dose and greater in individuals who were older at the time of the bombing (Kusunoki et al. 2003). This deviation might be associated with the presence of large clonal populations, since spectratyping of TCR Vβ genes for several A-bomb survivors who exhibited large deviations of memory CD4 T-cell Vβ repertoire showed that there were clonally expanded memory CD4 T-cell populations (unpublished observation). We suspect that A-bomb irradiation may have resulted in preferential expansion of memory CD4 T-cell clones that might have existed at the time of the bombing (Figure 2C), and have previously obtained evidence from a study of the progeny of a hematopoietic stem cell bearing a unique mutation that supports this plausible scenario (Kusunoki et al. 2002b). Such clonal expansions may be related to high (and perhaps overly prolonged) cytokine production in response to radiation-induced damage plus exposure to previously encountered or cross-reactive antigens that activate specific memory T cells during the time of excessive cytokine production. It is of course possible that confounding factors, such as infections or other stresses, had additional adverse effects on the maintenance of memory T-cell pools in the survivors. Thus, our current interpretation of long-lasting alterations in the T-cell system of survivors is that previous radiation exposures may have reduced the individuals' ability to produce new T cells and to maintain a fully diverse repertoire of helper T-cell memory.

As for memory CD4 T cells, significantly increased deviation of T-cell receptor repertoire of was observed only in survivors who were 20 or more years old of age at A-bomb exposures (Kusunoki et al. 2003), indicating different effects of irradiation depending upon the age at exposure (Figure 3). It is likely that memory CD4 T-cell pools of adults contain clonally expanded cell populations more frequently than those of children as a consequence of much more experiences of exposures to foreign antigens than children (Figure 2b). Restoration of memory CD4 T-cell pools would therefore have accompanied expansion of clonal populations, namely the deviation of T-cell receptor repertoire, more frequently in adults than children.

The perturbed T-cell homeostasis within A-bomb survivors seems to resemble that of normal aging people, i.e., reduction in the size of naïve T-cell pools and deviation in the repertoire of memory T-cell pools. Various studies using mice have also reported age-dependent decrements in the capacity of adult stem cells to repopulate T-progenitor cell pools (Hirokawa et al. 1992, Morrison et al. 1996), and to restore deficiencies in thymic functions that are involved in the production of mature T lymphocytes (Hirokawa et al. 1992, Mackall et al. 1998). It seems reasonable that such declines would tend to ensure that the restoration of peripheral T-cell pools becomes more dependent on the expansion of mature T cells in older hosts than younger ones (Hirokawa et al. 1992). Thus it can be argued that A-bomb irradiation accelerated the natural processes associated with immunological aging. Of particular interest is that a similar aging accelerated by A-bomb radiation has been postulated for cancer development (Pierce & Mendelsohn 1999).


A
Peripheral T-cell pool
Naive
Memory
Thymus
Education
Stem cells
Bone marrow
Response to pathogen A
(primary response)
Response to pathogen A
(secondary response)
B
Peripheral T-cell pool
Naive
Memory
Thymus
Reduced ability to
produce T cells
Impaired response
Pathogen X
Stem cells
Response to pathogen A
(secondary response)
C
A-bomb radiation
Peripheral T-cell pool
Naive
Memory
Thymus
Reduction in the
number of naïve cells
Reduced ability to
produce T cells
Impaired response
Pathogen X
Stem cells
Expansion of clonal
populations

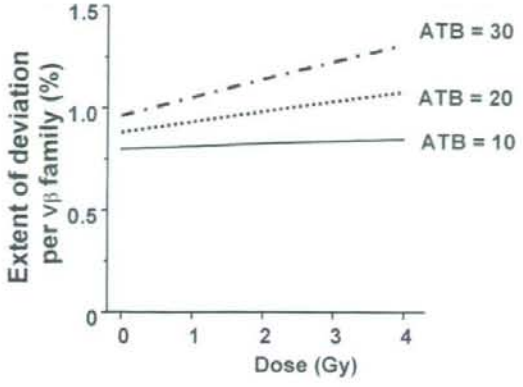


Figure 3. Evaluation of T-cell repertoire of memory CD4 T-cell population by determining to what extent any individual's value for the percentages of T cells expressing specific TCR Vβ families deviates from the average value for all subjects. The data in this figure are taken from those presented in a previous study (Kusunoki et al. 2003). A total 710 survivors were analyzed for their TCR Vβ repertoire deviations. The values have been adjusted to those for males who were 10, 20, or 30 years of age at the time of the bombing (ATB), and plotted against radiation dose. The T-cell receptor repertoire in the memory CD4 T-cell populations diverged significantly from the population average for counterpart families, especially in individuals who had been exposed to higher doses and were at least 20 years of age ATB ($p < 0.05$).

Does A-bomb radiation-induced damage of the immune system lead to disease development?

As most of these immunological effects are relatively small (change of a few percent per Gy of exposure) (Table I), it is difficult to envision, let alone prove, that such slight changes in the immune system could promote vulnerability to any particular disease. Nevertheless, these variations are markers of disease risk and do indeed serve to indicate individual's likelihood of contracting a given illness. They are not diagnostic by nature and do not indicate specific illness. Current understanding is that subclinical changes in the levels of some inflammatory parameters can be associated with increased risks of specific diseases, even when the changes are within normal ranges but significantly deviate from average values (Park et al. 2002, Cesari et al. 2003, Spranger et al. 2003). It is reasonable to assume that the more severe the aging and/or radiation-associated perturbations of individual's immune system are, the higher the disease risk will be for the individual (Figure 4).

Statistically significant associations between inflammatory biomarkers (leukocyte count, erythrocyte sedimentation rate, alpha 1 globulins, alpha 2 globulins, and sialic acid) and radiation dose have been reported in A-bomb survivors (Neriishi et al. 2001). To test whether defects in CD4 helper T-cell activities in A-bomb survivors are related to inflammatory responses, we measured levels of inflammatory cytokines and C-reactive proteins (CRP) in plasma samples from a group of survivors (Hayashi et al. 2003b). We found a strong positive correlation between IL-6 and CRP levels relative to radiation dose, and a negative correlation between plasma IL-6 or CRP level and the percentage of peripheral blood CD4 T cells. These results could be interpreted to mean that sub-clinical inflammatory status is associated with a decrease in the percentage of CD4 T-cells.

There is emerging evidence that inflammatory processes are important in the development of atherosclerosis (Ross 1999). The pathological evidence is strong and recent large-scale epidemiological

Figure 2. T-cell homeostasis is likely to be perturbed by aging and/or radiation exposure. Letters indicate T cells with different antigen specificities. (A) T-cell homeostasis involves the maintenance of a balance between renewal and death among the naïve and memory T-cell populations. The naïve T-cell pool is primarily maintained by the inflow of T-cell populations that have acquired diverse receptors for recognition of various peptides associated with self MHC molecules in the thymus (education). Once the immune system encounters an antigen, a population of T cells in the naïve T-cell pool will recognize the antigen and proliferate, but most of the cells that proliferate will die, with only a few entering the memory T-cell pool after the immune response has run its course (primary response). T cells in the memory pool can be recalled by antigens that have previously been encountered by the immune system (secondary response). A secondary response is usually more rapid and vigorous than a primary one. Although only a few memory T cells return to the memory pool after the secondary immune response has run its course, the overall pool for a specific antigen is now larger than it was after the primary response. (B) Our ability to maintain both naïve and memory T-cell pools is believed to decline with age. In older people, the naïve T-cell pool becomes reduced in size as a result of diminishing rates of production of new T cells in the thymus, so their response to antigens that have not previously been encountered (i.e., antigen X in this figure) begins to be impaired in comparison with those of younger individuals. Although fewer naive T cells move into the memory T-cell pool, the size of the memory T-cell pool is nonetheless constant even in aging individuals. However, some cells proliferate preferentially, and clonally expanded populations frequently appear to arise in the memory T-cell pools of older individuals. Thus, clonal populations often come to represent a considerable percentage of the memory T-cell pool, and this may lead to a distorted array of immune responses to antigens. (C) Perturbation of T-cell homeostasis in A-bomb survivors supposedly resembles that in aged persons. A-bomb radiation exposure may have damaged the ability of the thymus to produce naïve T cells and subsequently resulted in reduced size of the naïve T-cell pool; T-cell responses to antigens that have not previously been encountered (i.e., antigen X in this figure) may be associated with increased risk of infection-associated diseases and possibly with smoldering inflammation that may link to increased risk of particular diseases such as myocardial infarction. The maintenance of memory T-cell pool may have also been perturbed by A-bomb radiation exposure. Although the size of memory T-cell pool is not reduced by A-bomb radiation exposure, emergence of clonal expansions of a part of the memory T-cell population has frequently been observed in the memory T-cell populations of A-bomb survivors.

studies suggest that even small increases in CRP levels – accurate indicators of levels of inflammation – may be an important risk factor in inflammation, useful in predicting susceptibility to myocardial infarction (MI), stroke or peripheral arterial disease (Ridker et al. 1997, Koenig et al. 1999, Danesh et al. 2000, Mendall et al. 2000, Ridker et al. 2000, Ridker et al. 2001). To try to investigate the relationships between radiation-associated immunological alterations and diseases, we investigated whether or not any immunological changes in A-bomb survivors were associated with the pathogenesis of cardiovascular diseases, including MI. This investigation was based on studies indicating that inflammation plays a role in this type of cardiovascular disease. Furthermore, a radiation dose-dependent increase in relative risk of MI was observed in an A-bomb survivor cohort where extended, biannual health examinations have been conducted (Kodama et al. 1996a, Yamada et al. 2004). The prevalence of MI was significantly higher in individuals who had reduced CD4 T-cell percentages (Kusunoki et al. 1999), especially in those in which the size of naïve CD4 T-cell populations were relatively small compared to the average value (Kusunoki et al. 2002a) (Figure 5). It is therefore possible that the resulting reductions in naïve T-cell pool sizes might be related to certain inflammation-associated diseases in A-bomb survivors. As for inflammatory biomarkers, IL-6 levels were significantly higher in survivors with a history of MI than in those without such a history (Hayashi et al. 2003b) (Figure 6). A similar elevated trend in survivors with a history of MI was also

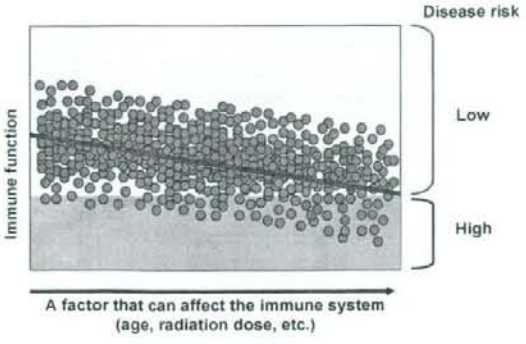


Figure 4. A schematic model showing how a slight immunological change may have caused an increased risk of disease. Each circle represents an individual value for any given immunological parameter, and the line shows the regression between value for the immunological parameter and a key environmental factor such as radiation dose. The lower the value for the immunological parameter an individual has come to possess as a consequence of aging and/or radiation exposure, the higher the disease risk of the individual.

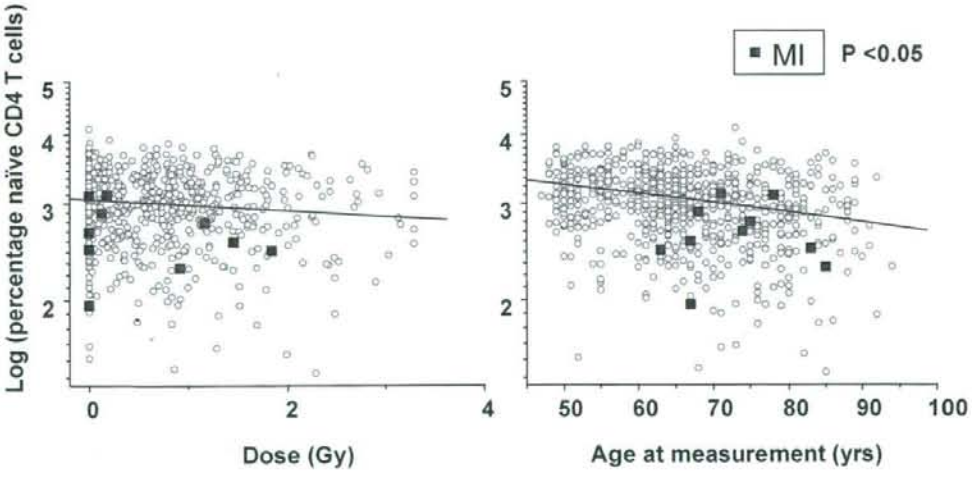


Figure 5. Proportion of peripheral blood CD45RA-positive naïve CD4 T cells in A-bomb survivors ($N = 723$) with histories of myocardial infarction (MI, closed larger symbols, $n = 10$) and those without such histories (open smaller symbols). Lines denote regression between logarithmically transformed naïve CD4 T-cell proportion and radiation dose (left panel) or age (right panel), after adjusting the proportions for 66-year-old male (left panel) or unexposed males (right panel), respectively. A standard multiple regression method was used to regress logarithmically transformed naïve CD4 T-cell proportion on age at examination, gender, radiation dose, and history of myocardial infarction. Estimated radiation doses were based on the 1986 Dosimetry System known as DS86; basically this involves calculating a free-in-air radiation dose estimate for the subject's reported location and then adjusting the value obtained to reflect shielding information (Roesch 1987). Naïve CD4 T-cell proportion is significantly ($p < 0.01$) higher among females than males and has decreased with age ($p < 0.01$) and dose ($p < 0.01$). Proportion of naïve CD4 T cells is significantly ($p < 0.05$) lower in survivors with myocardial infarction than in those without. This figure is a representation of results in a previous study (Kusunoki et al. 2002b).

found in levels of CRP (Hayashi et al. 2003b) (Figure 6). A plausible interpretation is that the decreased number of CD4 T cells may partly be linked to the low-grade inflammation indicated by increased levels of IL-6 and CRP. Such attenuation of T-cell immunity associated with long-lasting inflammation could lead to increased risk of certain diseases such as MI in A-bomb survivors (Figure 7). However, it is still possible that MI itself might be responsible for noted defects in CD4 T-cell population, and for the correlated increases in CRP and IL-6 levels. Subjects have been analyzed cross-sectionally and, hence, only the long-surviving individuals have been evaluated, and not the total 'at risk' population. Clearly prospective studies will be required to test these hypotheses directly.

Conclusions and perspectives of immunology studies on A-bomb survivors

In summary, A-bomb irradiation may have perturbed T-cell homeostasis, resulting in loss of T-cell immunity. Such abnormalities in the T-cell system may cause chronic inflammation, and in turn, be partly responsible for cardiovascular disease and other gerontological-associated diseases of importance. The following issues should be addressed in order to better understand, from an immunological point of view, how A-bomb radiation has biologically affected humans and has caused numerous diseases. In this regard it is important to directly address whether A-bomb irradiation has accelerated immunological aging by perturbing T-cell homeostasis; e.g., to confirm the increased rates of age-dependent thymus dysfunction and the T-cell telomere length shortening in exposed individuals. Longitudinal analyses of the change in the various immunological parameters will provide a suitable vehicle in better understanding immunological aging in the A-bomb survivors.

It is also important to characterize and document these temporal, perhaps causal relationships between radiation-induced perturbations of T-cell homeostasis and chronic inflammation leading to various diseases in A-bomb survivors. Data obtained from comparative, periodic measurements of serum

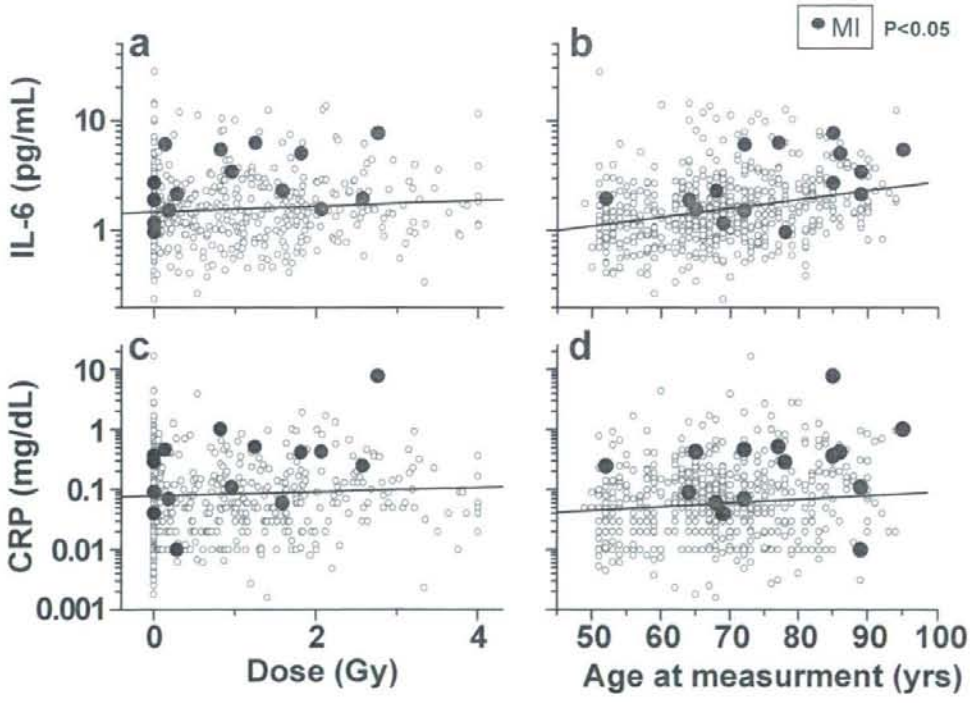


Figure 6. Plasma IL-6 (a, b) and CRP (c, d) levels in 453 A-bomb survivors with histories of MI (closed larger symbols, $n = 12$) and those without such histories (open smaller symbols). Lines denote regression lines between IL-6 level and radiation dose (a) or age (b), and between CRP level and radiation dose (c) or age (d): $\log(level) = \alpha + \beta_1(gender) + \beta_2(age) + \beta_3(dose)$, where $gender = 0$ for male and $= 1$ for female. IL-6 and CRP levels increase with radiation dose ($p < 0.01$), and there were age-dependent increases in both levels ($p < 0.01$). The association between a history of MI and IL-6 or CRP level was analyzed based on a multiple logistic model to adjust for gender, age, and DS86 radiation dose. The levels are significantly ($p = 0.05$) higher in survivors with myocardial infarction than in those without. This figure is a representation of results in a previous study (Hayashi et al. 2003b).

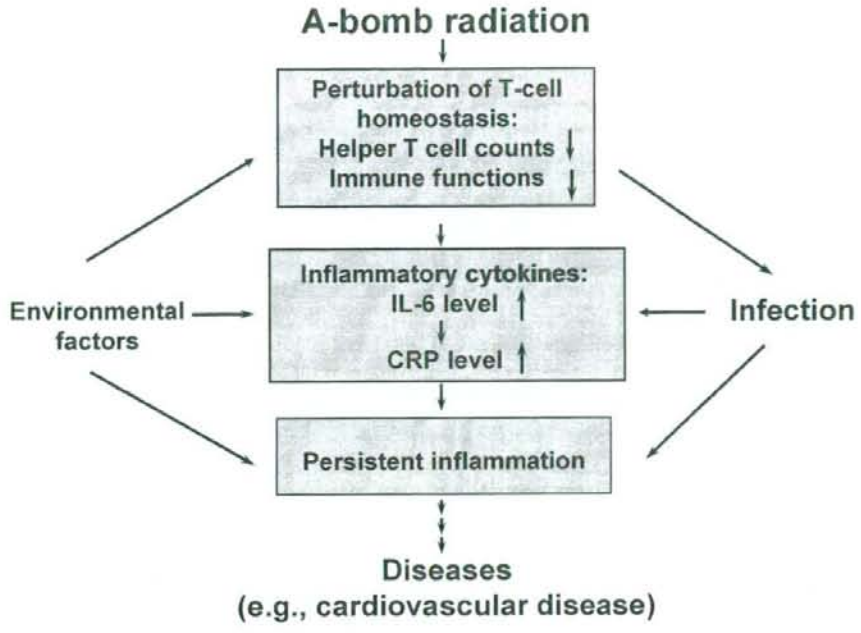


Figure 7. A possible immunological mechanism playing a part in disease development in A-bomb survivors. A-bomb radiation may have perturbed T-cell homeostasis, resulting in deficits of helper T-cell counts that are associated with reduced immune functions. Such abnormalities in the T-cell system may cause long-lasting inflammation that could lead to the development of, e.g., cardiovascular diseases. Infections and other environmental factors such as lifestyle may further interact with the process of disease development.

cytokine levels and surface markers of lymphocyte subsets in survivors, relative to the onsets of various diseases, will be used as a longitudinal response assessment tool, prospectively and retrospectively. In addition, it is quite apparent that there are large individual variations in the levels of immunological and inflammatory markers (e.g., see Figures 5 and 6): Not all individuals who show reduced immune functions and/or elevated inflammatory biomarkers develop particular diseases. It is also well known that both immune and inflammatory responses are controlled by an array of polymorphic genes. Thus, differences in genetic backgrounds are likely to underlie individual differences in disease susceptibility. Our preliminary study on a group of A-bomb survivors in Hiroshima suggests the possibility that prevalence of type-2 diabetes may be affected by radiation dose in individuals with a particular human leukocyte antigen (HLA) type but not in individuals with the other HLA types (Hayashi et al. 2003a). Such an immunogenetic approach would very likely provide new insights into determining the mechanisms by which acute, ionizing radiation exposure causes disease. A finding based on genetic differences between individuals would be more revealing than one based on conventional phenotype differences. If genetic differences within the immunogenome can explain differences in disease susceptibility, then it seems reasonable to suggest that there is an immunological mechanism involved in the development of this particular disease and perhaps others.

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