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Radiation- and Age-Associated Changes in Peripheral Blood Dendritic Cell Populations among Aging Atomic Bomb Survivors in Japan

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Previous immunological studies in atomic bomb survivors have suggested that radiation exposure leads to long-lasting changes, similar to immunological aging observed in T-cell-adaptive immunity. However, to our knowledge, late effects of radiation on dendritic cells (DCs), the key coordinators for activation and differentiation of T cells, have not yet been investigated in humans. In the current study, we hypothesized that numerical and functional decreases would be observed in relationship to radiation dose in circulating conventional DCs (cDCs) and plasmacytoid DCs (pDCs) among 229 Japanese A-bomb survivors. Overall, the evidence did not support this hypothesis, with no overall changes in DCs or functional changes observed with radiation dose. Multivariable regression analysis for radiation dose, age and gender effects revealed that total DC counts as well as subpopulation counts decreased in relationship to increasing age. Further analyses revealed that in women, absolute numbers of pDCs showed significant decreases with radiation dose. A hierarchical clustering analysis of gene expression profiles in DCs after Toll-like receptor stimulation *in vitro* identified two clusters of participants that differed in age-associated expression levels of genes involved in antigen presentation and cytokine/chemokine production in cDCs. These results suggest that DC counts decrease and expression levels of gene clusters change with age. More than 60 years after radiation exposure, we also observed changes in pDC counts associated with radiation, but only among women.

Editor's note. The online version of this article (DOI: 10.1667/RR14854.1) contains supplementary information that is available to all authorized users.

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INTRODUCTION

It is well known that ionizing radiation exposure can cause damage and compromise the human immune system, leading not only to acute (short-term) symptoms, but also to late (long-term) adverse effects. Late radiation effects on immunity have been studied in atomic bomb (A-bomb) survivors at the Radiation Effects Research Foundation (RERF), Japan (*1*). Previously published immunological studies have revealed that immunological alterations resulting from atomic bomb radiation exposure include a decrease in naïve T cells and an increase in memory T cells (*2*), skewed repertoire of T-cell receptor Vβ families (*3*), clonal T-cell expansion indicated by chromosomal aberrations (*4*) and increased plasma levels of reactive oxygen species and cytokines such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) with rising radiation dose (*5, 6*). These immunological alterations resemble the micro-immunological changes that occur with aging in individuals not exposed to radiation (*7, 8*). Based on those observations, we hypothesized that radiation exposure accelerates immunological aging, namely ''immunosenescence'', in A-bomb survivors.

Immunosenescence affects not only adaptive immune responses (T- and B-cell responses), but also innate immune responses. Dendritic cells (DCs) are one type of innate immune cell known for antigen presentation, being key components linking innate and adaptive immunity through priming of naïve T cells and shaping adaptive T-cell responses. There are two major DC subsets in human peripheral blood: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). These DC subsets recognize different pathogen-associated molecular patterns by expressing distinct repertoires of Toll-like receptors (TLRs) and other receptors. Engagement of specific DC subset TLRs in turn triggers distinct immune response pathways. For example, pDCs produce a large amount of interferon alpha (IFN-α) in response to virus infection (*9*). Age effect studies of human DCs have been controversial. While some studies report

age-dependent declines, others demonstrate no difference in the number of circulating DCs (*9–14*). Several reported studies have also indicated age-related functional changes in DCs, such as impaired expression of TLRs (*13*), decreased production of cytokines, chemokines and IFN-α after TLR-stimulation (*9, 10, 12, 13, 15, 16*) and increased responses to self-antigen (*15*). Thus, the assessment of aging-related changes in DCs is believed to contribute to the understanding of aging-induced detrimental effects on the immune system, such as age-related changes in the functional diversity of effector T-cell subsets, resulting in increased risks of autoimmune diseases and chronic inflammatory diseases.

Radiation-induced changes in DCs are not well understood. *In vitro* investigations showed that high-dose radiation (i.e., 10–20 Gy) induced alterations in functions of both human and mouse DCs, such as inefficient antigen presentation activity (*17, 18*), low capacity to induce T-cell proliferation (*19*) and reduced co-stimulatory receptor expression (*19, 20*). However, the effects of radiation on DCs are likely radiation dose-dependent. For example, acute high-dose whole-body irradiation (i.e., 10–20 Gy) tends to suppress DC functions, but effects of low-dose irradiation (i.e., 1 Gy or less) are reportedly contradictory and still a matter for discussion (*21, 22*). To our knowledge, regarding late effects of radiation on the human immune system, DCs have not been evaluated; and thus, there has been no evidence for long-term DC alterations associated with radiation exposure to non-lethal doses.

In the current study, we evaluated the long-term radiation effects on circulating DCs in peripheral blood obtained from A-bomb survivors in Japan, with the hypothesis that A-bomb radiation exposure induced premature aging of DCs, resulting in reduced numbers and impaired functions of DCs. We report the novel observation that the effects of past radiation exposure persist in DCs of A-bomb survivors who were exposed 65 years ago.

MATERIALS AND METHODS

Study Participants

Selection of participants for this study followed a similar protocol to that previously described elsewhere (*23*). Participants were selected for this cross-sectional study from 1,705 Hiroshima A-bomb survivors participating in the Adult Health Study (AHS) at RERF. Estimates for radiation exposure to the bone marrow were used, where calculations were based on the revised DS02 A-bomb radiation dosimetry (DS02R1); weighted absorbed bone marrow doses with a neutron-relative biological effectiveness of 10 were used (*24*). Of these participants, we excluded the following individuals: 1. Those who had medical histories of serious immune-related diseases [Supplementary Table S1 (criteria I); http://dx.doi.org/10.1667/RR14854.1.S1]; 2. Those who had ever experienced radiation therapy, interferon treatment or erythropoietin treatment; 3. Those who had either received steroid treatment or been diagnosed with a solid cancer within five years prior to the DC examination; 4. Those who were currently diagnosed with selected benign diseases that potentially affected the immune system [Supplementary Table 1 (criteria II)]; or 5. Those who were older than 90 years on June 30, 2011. Between July 2011 and December 2013, we obtained blood samples with written informed consent from 243 participants selected using a stratified random sampling method for the sex, radiation dose and age category strata. Fourteen participants were excluded from the data analysis due to an update of their disease status after blood collection. The final analysis dataset consisted of data from 229 participants with evaluable samples (Supplementary Table S2; Fig. 1).

Ethics Statement

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

Enumeration and Isolation of DCs from Peripheral Blood

Procedures for cDC and pDC collection are shown in Fig. 1A. A volume of 4–8 ml venous blood was collected from each participant into a 15-ml polypropylene tube (IWAKI; Asahi Glass Co. Ltd., Chiba, Japan) containing 50 units of novo-heparin (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). Peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll density gradient centrifugation (Lymphocyte Separation Medium 1077; Wako Pure Chemical Industries Ltd., Osaka Japan) within 4 h after blood collection. PBMCs were stained with the following fluorescently-labeled antibodies: PE-conjugated anti-CD11c (clone, B-ly6; BD Biosciences, Franklin Lakes, NJ), APC-conjugated anti-CD123 (clone, AC145; Miltenyi Biotec, Bergisch Gladbach, Germany), PerCP-conjugated anti-HLA-DR (clone, L243; BD Biosciences), FITC-conjugated lineage markers (Lin); anti-CD3, anti-CD14, anti-CD16 (clones, 7D6, TKü4, 3G8; Invitrogen™, Carlsbad, CA), anti-CD19 anti-CD20 (clones, HIB19, 2H7; BD Biosciences) and anti-CD56 (clone, MEM188; e-Bioscience™/Thermo Fisher Scientific™ Inc., Rockford, IL). For this cell staining, the PBMCs were centrifuged in a 15-ml polypropylene tube and incubated with Lin (7.5 μl/10^6 cells), anti-CD11c (10 μl/10^6 cells), anti-HLA-DR (10 μl/10^6 cells) and anti-CD123 (1 μl/10^6 cells) for 30 min on ice in the dark. Stained cells were then washed with PBS containing 5 m*M* EDTA and 1% fetal bovine serum (FBS). According to the gating strategy shown in Fig. 1B, cDCs ($CD11c^+HLA\text{-}DR^+Lin^-$) and pDCs ($CD123^+HLA\text{-}DR^+Lin^-$) were enumerated and sorted using a FACS (J-SAN; Bay Bioscience, Kobe, Japan). Cell sorting was performed at 4°C with a cooling apparatus, SCA-11 (Sanyo, Osaka, Japan). Absolute numbers of total DCs, cDCs and pDCs were estimated by multiplying the percentages of DC subsets in PBMCs by lymphocyte and monocyte counts, which were determined in the blood using an automated hematology analyzer (Sysmex XN-1000; Sysmex Corp., Kobe, Japan).

In Vitro TLR Stimulation of Sorted DC Subsets

In addition to evaluating the influence of radiation dose, age and sex on peripheral DC frequency and absolute number, we also assessed DC subset function by evaluating surrogate markers of function by *ex vivo* stimulation. Cells and culture supernatant were collected with and without DC subset-specific TLR stimulation and markers related to DC function, such as antigen presentation and production of cytokine/chemokines, and transcription factors involving TLR signaling pathways were determined by quantitative (q) reverse transcriptase (RT) PCR arrays or multiplex luminex cytokine protein arrays. The primary end points were gene expression profiles and related biological pathways. Secondary end points were *in vitro* cytokine production profiles. Sorted cDCs and pDCs were cultured for 4 h in the presence or absence of TLR ligands Poly(I:C) (10 μg/ml; InvivoGen, San Diego, CA) and R848 (10 μ*M*; imidazoquinoline; Enzo Life Sciences, Farmingdale, NY), respectively, in 96-well plates (Corning® Inc., Corning, NY) supplemented with RPMI1640 medium

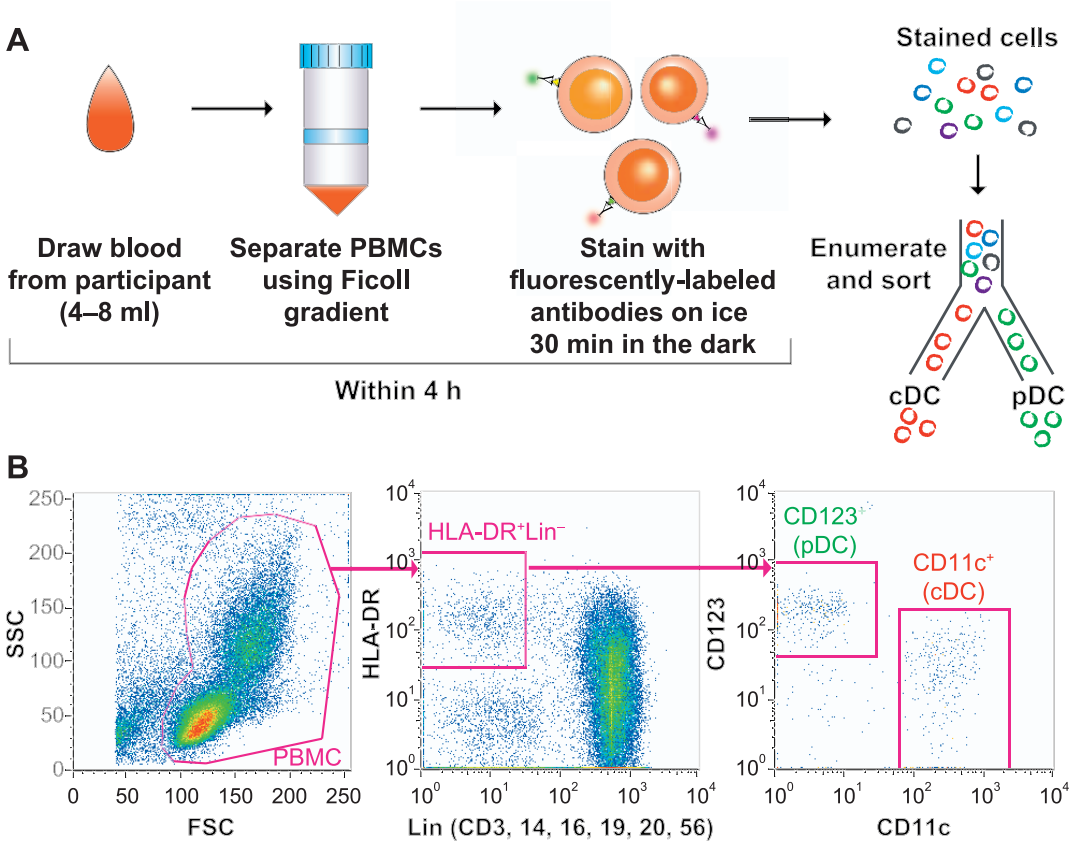

FIG. 1. Outline of DC isolation. Panel A: Peripheral blood was drawn (4–8 ml) into heparinized tubes from each participant. PBMCs were isolated using Ficoll density gradient centrifugation within 4 h of the blood draw. Freshly-isolated PBMCs stained with fluorescently-labeled antibodies were sorted into cDCs ($CD11c^+HLA\text{-}DR^+Lin^-$) and pDCs ($CD123^+HLA\text{-}DR^+Lin^-$). Panel B: Gating strategy for enumeration and sorting of the DC populations. Flow cytograms represent one example. FSC = forward scatter change; SSC = side scatter change.

(Nacalai Tesque Inc., Kyoto, Japan) containing 10% FBS (HyClone™ Laboratories, Logan, UT.), 2 m*M* L-glutamine (Gibco®/Life Technologies, Grand Island, NY) and 1% penicillin/streptomycin (Gibco), at 37°C in a humidified atmosphere flushed with 5% CO_2. For gene expression assays, total RNA was isolated from 2,500 cDCs and pDCs each, and were similarly cultured for 4 h in the presence or absence of PolyI:C and R848, respectively. For multiplex cytokine/chemokine analysis, 5,000 cDCs and pDCs each were cultured for 48 h in the presence or absence of PolyI:C and R848, respectively. Collected culture supernatants were stored at –80°C until their use for multiplex cytokine/chemokine analysis.

Gene Expression Assay by PCR Array

Expression levels of 96 genes were determined using a custom human Dendritic and Antigen presenting cell RT^2 Profiler PCR array (SABiosciences, Frederick, MD), which included 85 key genes involved in the TLR signaling pathway and controls. Total RNA was extracted from TLR-stimulated and TLR-unstimulated DCs using PicoPure RNA isolation kit (Applied Biosytems®, Foster City, CA). cDNA was synthesized from total RNA using RT^2 PreAMP cDNA synthesis kit (QIAGEN, Hilden, Germany). Real-time PCR was performed in 96-well plates using RT^2 SYBR® Green qPCR Master Mix (SABioscience) in a CFX96 Real Time PCR Detection System (Bio-Rad® Laboratories Inc., Hercules, CA). Ct values were obtained using a constant baseline threshold for all PCR runs. Four endogenous expression controls provided by the array (ACTB, B2M, RPL13A, UBE2N) were used to calculate the arithmetic mean which was then set as the Ct value for normalization. The relative amount of target genes in each sample was calculated in comparison with the calibrator sample using the delta-delta threshold cycle (ΔΔCt) method (*25*). The magnitude of gene induction was calculated using the formula $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ for unstimulated cells} - \Delta Ct \text{ for stimulated cells})}$.

Multiplex Cytokine/Chemokine Analysis

Cytokine/chemokine profiling of TLR-unstimulated and TLR-stimulated DC culture supernatants was performed using a Luminex® bead-based human 25-plex cytokine assay (Life Technologies) according to the manufacturer's protocol using overnight primary incubation periods. Multiplex Luminex assays were run by the Duke Human Vaccine Institute Regional Biocontainment Laboratory Immunology Unit (Durham, NC). The final dataset consisted of cytokine/chemokine data for both cDCs (n = 215 participants; 73 men and 142 women) and pDCs (n = 163 participants; 63 men and 100 women). Each sample was evaluated in singlet. Final observed pg/ml of each analyte in the assay was quantified using a 5PL curve fit of assay kit standards using Bio-Plex® Manager software (Bio-Rad Laboratories). The lower limit of quantification (LLOQ) for each analyte per run was set to the lowest valid point on the standard curve. Sample values at or below the LLOQ were replaced with a value equal to one-half the LLOQ (i.e., midpoint). Those values identified as above the upper limit of quantification (ULOQ; the highest valid point on the standard curve) were set equal to the analyte- and plate-specific ULOQ.

TABLE 1
Characteristics of Study Participants

	Non-exposed (<5 mGy)	Low dose (5–500 mGy)	High dose (≥500 mGy)	*P* value*
Age at blood sampling				
<80 years	49	30	58	0.58
≥80 years	34	23	35	
Median (range)	79 (66–90)	80 (66–88)	79 (66–90)	0.8
Sex				
Men (n = 76)	27	20	29	0.73
Women (n = 153)	56	33	64	
Radiation dose (mGy)				
Median (range)	1 (0–2)	132 (12–487)	983 (504–3,802)	

* *P* values are based on the Kruskal-Wallis nonparametric test.

Statistical Analysis

We evaluated measurements for the total dendritic cell (total DC), conventional DC (cDC) and plasmacytoid DC (pDC) populations across all participants as well as within male and female cohorts. These markers were descriptively and graphically summarized overall and in relationship to radiation dose, age and sex. Differences in the various DC measurements were compared between groups using Wilcoxon rank sum tests or Kruskal-Wallis tests, as appropriate. Linear regression models were used both in the univariate and multivariable setting to evaluate the influence of radiation dose, age and sex on DC measurements, with transformations used as necessary to achieve approximately normal distributions.

We characterized gene expression profiles of target genes using a hierarchical clustering analysis (11 control genes were removed). Target genes with fold changes less than 2 and more than 1/2 among 90% of participants were excluded (7 and 2 genes from the cDC and pDC analyses, respectively). In addition, we excluded 10 and 7 participants from cDC and pDC analysis, respectively, based on manufacturer quality-control metrics, such as test for PCR reproducibility, reverse transcription efficiency and the presence of genomic DNA contamination. Gene expression data were therefore available for 78 genes among 210 participants (66 men and 144 women) in cDC and for 83 genes among 213 participants (71 men and 142 women) in pDC. Clustering was performed on the Z-scaled expression levels with the Ward method, based on squared Euclidean distance (*26*). First, we identified clusters of differentially expressed genes. We then identified clusters of participants according to their gene expression cluster. A multivariable logistic regression model compared the radiation dose, age and sex between these clusters of participants. Scaling and clustering was conducted using statistical package R (The R Foundation, https://cran.r-project.org/), including the genefilter extension package (*27*).

Multiplex cytokine/chemokine protein profiling assays were analyzed by calculating the stimulation effect, calculated as the stimulated minus the unstimulated sample analyte level (pg/ml). As noted above, those with levels below the lower limit of quantification (LLOQ) or with estimates below this level were all imputed to have a value at the midpoint between 0 and the plate-specific LLOQ for that marker. We evaluated the robustness and sensitivities of the analyte measurements themselves by DC cell type (cDC or pDC). In evaluating the full panel of 25 cytokine/chemokine markers, we identified those markers that had at least 20% of stimulated samples with values calculated above the LLOQ. There were 14 markers that were excluded, which had more than 90% of participants with measurements below the LLOQ, with 12 of these markers having all participants with stimulated measurements below the LLOQ. Overall, 11 markers met these criteria: IFN-α, IL-6, RANTES, MIP-1α, MIP-1β, MCP1, TNF-α, IL-1Ra, IP-10, IL-2Ra and IL-8. Cytokine production levels (pg/ml in culture supernatant) were compared between clusters using logistic regression models, adjusted for radiation dose, age and sex.

RESULTS

Demographic characteristics of all participants and their distribution among three dose-based subsets (<5, 5–500 and ≥500 mGy) are summarized in Table 1. Nearly two-thirds of all participants were women (n = 153), and the median age at the time of blood sample collection was 79 years (range: 66 to 90 years). There were no significant differences in distribution of age or sex across the dose groups (Table 1).

Numerical Changes in Circulating DCs among A-Bomb Survivors

Total DC number as well as the estimated absolute numbers and frequencies of cDCs and pDCs in PBMCs for all 229 participants in the cohort are summarized in Table 2.

TABLE 2
Enumeration of DC Subsets

Median (range)		Total DCs (n = 229)	cDC (n = 229)	pDC (n = 229)
Absolute number (per ml)				
All participants	(n = 229)	13.0 (2.5–34.6)	8.8 (1.9–26.4)	4.0 (0.3–12.9)
Men	(n = 76)	15.3 (2.5–26.6)	9.6 (21.0–2.2)	4.8 (0.3–11.7)
Women	(n = 153)	12.6 (3.3–34.6)	8.6 (1.9–26.4)	3.5 (0.3–12.9)
Percentage of PBMCs				
All participants	(n = 229)	0.64 (0.17–1.54)	0.45 (0.07–1.15)	0.18 (0.01–0.62)
Men	(n = 76)	0.70 (0.17–1.54)	0.47 (0.15–1.15)	0.21 (0.02–0.62)
Women	(n = 153)	0.63 (0.18–1.42)	0.44 (0.07–0.99)	0.17 (0.01–0.59)

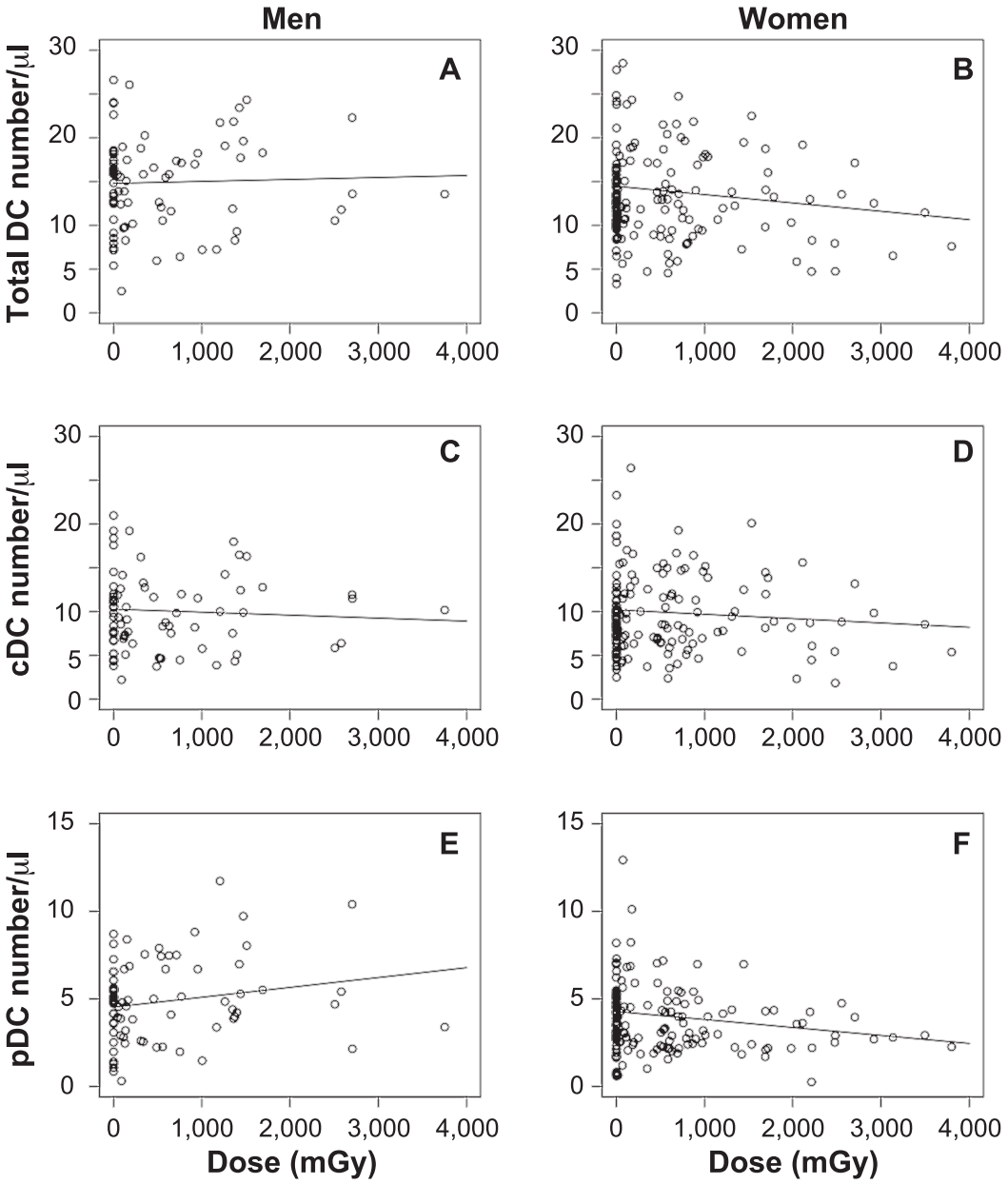

FIG. 2. Radiation dose-dependent differences in absolute numbers of DC subtypes. Scatterplots of radiation dose versus absolute numbers of DC subtypes by sex. Results in men and women, respectively, for total DCs (panels A and D), cDCs (panels B and E) and pDCs (panels C and F) indicate that in men (n = 76), there were no significant effects of radiation dose on the absolute numbers of DC subtypes, and in women (n = 153), the absolute numbers of total DCs ($P = 0.048$) and pDCs ($P = 0.035$) decreased with increasing radiation dose. The fitted line reflects a linear regression curve fit to the data.

Total DC numbers (per μl) ranged from 2.5–34.6 (median = 13.0) in this aged population. The absolute cDC and pDC numbers (per μl) ranged from 1.9–26.4 (median = 8.8) and 0.3–12.9 (median = 4.0), respectively.

Dose-related difference. We first evaluated dose-related numerical changes in peripheral circulating DCs. Relationships between radiation dose and circulating peripheral DCs were evaluated in the multivariable setting adjusting for age at blood sampling (Fig. 2). In particular, we identified sex as a significant effect modifier on the relationship of radiation dose on pDC numbers ($P = 0.02$). To evaluate this further, we analyzed the dose effects on absolute pDC number (per 1 μl blood) in each sex. The results indicated that the absolute number of pDC in women revealed a significant inverse relationship with increasing radiation dose ($r = -0.17$, $P = 0.035$). However, there was no significant association observed in men between pDCs and radiation dose ($r = 0.12$, $P = 0.32$). Also, the absolute number of cDC, total DCs and measurements of cDCs and pDCs as a percentage of the total DC number did not appear to be

correlated with radiation dose in either sex. Thus, no radiation dose response was observed for total DCs or cDCs. The ratio of cDCs or pDCs to total DCs ranged from 0.05–0.62 (median = 0.30) or from 0.38–0.95 (median = 0.70), respectively, but did not correlate with radiation dose ($r = 0.01$ and $P = 0.87$ for both the radios). Also, the pDC to cDC ratio ranging from 0.06–1.67 (median = 0.42) did not correlate with radiation dose ($r = 0.01$, $P = 0.93$). A decrease in the absolute number of pDCs with radiation dose was observed in women but not in men.

Sex difference. We further analyzed DC measurements in relationship to sex. Total DC numbers were significantly higher in men than women (medians: 15.3 vs. 12.6, respectively; $P = 0.034$). We further observed a tendency towards higher pDC numbers in men than women (medians: 4.8 vs. 3.5, respectively; $P < 0.001$), whereas no significant sex-based differences were observed for cDC numbers (medians: 9.6 vs. 8.6, respectively; $P = 0.61$). The same patterns of relationships were observed for the percentage markers of these DC measurements between men and women, indicating that the numerical sex difference in circulating DC populations is mainly ascribed to higher number of pDCs but not cDCs in men.

Age-related difference. It has been reported that numbers of both adaptive and innate immune cells decline with age. Thus, we next evaluated age-related numerical changes in peripheral circulating DCs in our cohort (Fig. 3). Consistently for all DC measurements, age was an influential factor both in the univariate setting as well as when radiation dose and sex were included in the models. As expected, each of these DC measurements had an inverse correlation with age ($r = –0.20$, $P < 0.006$ for each). When participants were divided by sex, men did not show significant inverse correlations with age: cDCs ($r = –0.20$, $P = 0.084$), pDCs ($r = –0.003$, $P = 0.98$) and total DCs ($r = –0.15$, $P = 0.19$). Conversely, significant negative correlations were observed in women between age and total DC, pDC and cDC ($r = –0.23$, $r = –0.26$, $r = –0.17$; $P = 0.002$, $P = 0.004$, $P = 0.033$, respectively). The analysis of DC subset percentages in PBMCs also yielded similar results. Even with the considerably elderly skewed cohort (ages ranging from 66 to 90 years) compared to cohorts in other reported studies (*14*), our data still reveal age-associated attenuation of DCs, especially in women.

In Vitro Functional Analysis

TLR-induced gene expression profiles. We further investigated whether functions of DC subsets altered depending on past exposure, age or sex among survivors, by evaluating surrogate markers of DC functions after *ex vivo* TLR stimulations. First, to identify genes showing similar expression patterns, gene expression data for 78 genes in Poly I:C-stimulated cDCs were hierarchically clustered among all participants, based on the Ward method. Two major clusters of genes showing different gene expression patterns were identified (Supplementary Fig. S2A; http://dx.doi.org/10.1667/RR14854.1.S1). Furthermore, the gene expression pattern visualized by a heatmap (Supplementary Fig. S2B) delineated two major clusters of the participants, which shows different gene expression patterns that were identified in the group containing 45 genes. One cluster included 45 genes encoding the DC maturation and antigen presentation (*CD40*, *CD80*, *CD86*), antigen uptake (*CDC42*, *TAP2*; antigen peptide transporter 2), cytokine and chemokines (*CCL2*; MCP-1, *CCL3*; MIP-1α, *CCL3L*; C-C motif chemokine ligand 3 like 1, *CCL4*; MIP-1β, *CCL5I*; RANTES, *CCL7*; MCP-3, *CCL8*; MCP-2, *CCL13*; MCP-4, *CCL19*; MCP-3β, *CXCL1*; GROα, *CXCL10*; IP-10, *IL12A*, *IL12B*, *IL1A*, *IL1B*, *IL6*, *CXCL8*), cytokine and chemokine receptor and ligands (*CCR5*), dendritic cell differentiation (*CSF2*; GMCSF, *LYN*), interferon regulatory factor pathway (*IFIT3*, *IFNB1*; IFNβ, *IRF1*), inflammation (*PTGS2*; COX-2), NFκB pathway (*NFKB1*; NFκB p105, *NFkBIA*; IκBα, *MAP4K4*, *TAP2*), PI3K-AKT-signaling pathway (*EIF2AK2*; PRKR), TGF-β superfamily (*INHBA*, *TGFB1*; TGF-β1), TNF superfamily (*LTA*; TNFSF1, *TNF*; TNFα), TLRs (*TLR1*, *TLR2*, *TLR7*, *TLR4*) and TLR-signaling pathway (*HSPA1A*; HSP72, *PELI1*, *REL*; C-Rel, *MYD88*, *RIPK2*).

We then sought to identify clusters of participants with differential levels of gene expression. Additional hierarchical clustering was performed on the expression levels of these 45 genes. Two clusters of participants were identified: 97 participants in cluster 1 and 113 participants in cluster 2 (Fig. 4A). These clusters differed according to Poly I:C stimulation: cluster 1 exhibited a low response, while cluster 2 exhibited a high response. There was no difference in the sex distribution between clusters (70% vs. 67% women in cluster 1 vs. 2, $P = 0.77$). The median (interquartile range) radiation dose was 132 (1, 701) and 158 (1, 868) mGy among clusters 1 and 2, respectively (Fig. 4B). After adjustment for age and sex, the radiation dose distribution did not differ between the clusters ($P = 0.53$). The median (interquartile range) age was 80 (72, 83) years and 78 (71, 81) years among clusters 1 and 2, respectively (Fig. 4C). After adjustment for sex and radiation dose, the age distribution differed between the clusters ($P = 0.048$). Note that age and radiation dose were not correlated (Spearman rank correlation, –0.05; $P = 0.50$). After segregating changes in expression levels, after Poly I:C stimulation, we found older participants had lower expression levels than younger participants. In addition, the cluster included CD40, CD80 and CD86, which are all involved in DC maturation and antigen presentation. However, expression levels of this cluster were not associated with A-bomb radiation exposure more than 60 years ago.

We also analyzed pDC gene expression profiles that stimulated with R848 from 213 participants. The clustering procedure failed to identify clusters of differentially expressed genes (Supplementary Fig. S3; http://dx.doi.org/10.1667/RR14854.1.S1). *IFNA1* and *IFNB1* genes were

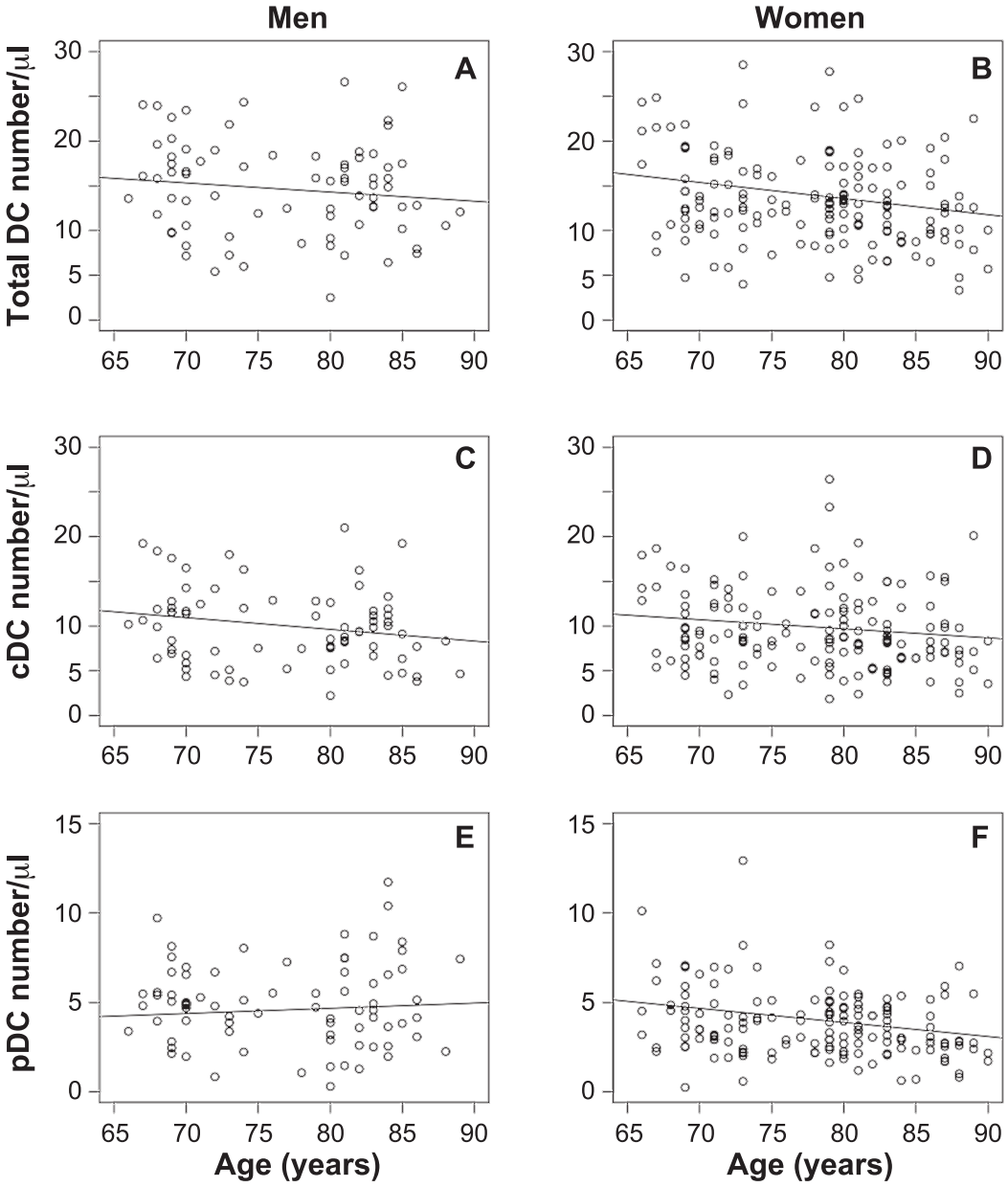

FIG. 3. Age-dependent differences in absolute numbers of DC subtypes. Scatterplots of age (age at blood collection) versus the absolute numbers of DC subtypes by sex. Results in men and women, respectively, for total DCs (panels A and D), cDCs (panels B and E) and pDCs (panels C and F) indicate that in men (n = 76), there were no significant effects of age on the absolute numbers of DC subtypes, and that in women (n = 153), the absolute numbers of total DCs ($P = 0.005$), cDCs ($P = 0.043$) and pDCs ($P = 0.004$) decreased with increasing age. The fitted line reflects a linear regression curve fit to the data.

upregulated with R848 stimulation; however, there were no associations with dose and age (data not shown).

TLR-induced cytokine/chemokine profiles. To further evaluate dose and age effects on DC subset function, we measured levels of secreted cytokines/chemokines produced *in vitro* by TLR-stimulated cDC and pDC. Eleven of the 25 analytes detected in the multiplex cytokine assay, IFN-α, IL-6, RANTES, MIP-1α, MIP-1β, MCP1, TNF-α, IL-1Ra, IP-10, IL-2Ra and IL-8, were sufficiently quantifiable for data analysis (see ''Materials and Methods''). In looking at the cytokine/chemokine markers in relationship to the cDC and pDC components, in the univariate nonparametric setting, none of these markers were significantly differentially expressed between men and women.

We next evaluated dose-related cytokine/chemokine production changes in differences between TLR simulated and unstimulated in each DC subset, in the multivariable setting adjusting for age. In cDC cultures, only MCP1 ($r = 0.24$; $P = 0.046$) and IL-8 ($r = 0.21$; $P = 0.071$) showed significant and borderline-significant increases with dose in men, respectively. In pDC-derived chemokines/cytokines, the production of MCP1, IP-10, MIP-1β and IL-6 were

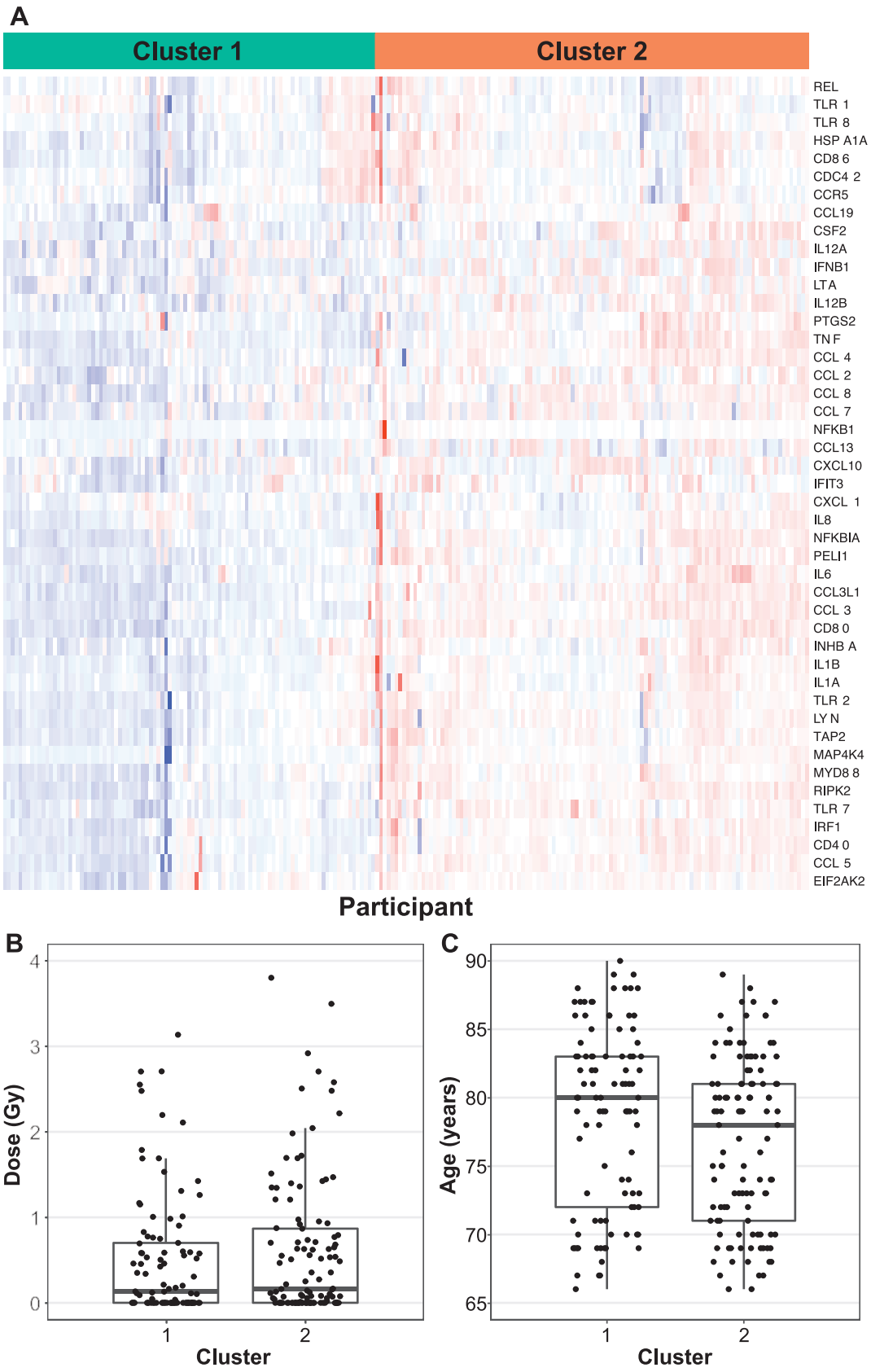

FIG. 4. Heatmap identifying two clusters of participants according to gene expression levels. Hierarchical clustering analysis of 45 genes expression in Poly I:C stimulated cDCs. Red and blue indicate upregulated and underexpressed genes, respectively (panel A). Boxplots exhibit the distribution of bone marrow dose (panel B) and age (panel C) between the clusters.

TABLE 3
Cytokine Production Levels were Compared between Clusters

	Cluster 1 (n = 97) Mean (SD)	Cluster 2 (n = 113) Mean (SD)	*P* value*
IFN-α	0.51 (3.7)	0.13 (9.2)	0.68
IL-1RA	3.7 (35)	14 (59)	0.13
IL-2R	–0.09 (7.5)	2.7 (13)	0.1
IL-6	5.2 (22)	18 (38)	0.022
IL-8	120 (1,300)	680 (1,700)	0.021
IP-10	2.9 (8.1)	11 (14)	<0.001
MCP-1	0.37 (18)	0.96 (42)	0.88
MIP-1α	6.6 (37)	23 (62)	0.052
MIP-1β	11 (27)	24 (42)	0.025
RANTES	1.0 (5.4)	3.6 (9.9)	0.062

* Adjusted for age, sex and weighted absorbed bone marrow dose.

dose-dependently increased in women but not men ($P = 0.019$, $P = 0.027$, $P = 0.051$ and $P = 0.064$, respectively). These results are partly concordant with the results from a multivariable analysis for age and radiation dose effects showing age-associated decreases in IL-6 ($P = 0.032$) and RANTES ($P = 0.02$) in women, and both significantly negative associations with age. pDC-derived MIP-1β production was inversely associated with age in women ($P = 0.041$).

Finally, we analyzed associations of the cDC cytokine/chemokine protein production levels from cDC with the clusters that were identified by gene expression analysis (Fig. 4). Our analysis revealed that after adjustment for radiation dose, age and sex, the amounts of IL-6, IL-8, IP-10, MIP-1α, and MIP-1β were significantly higher in cluster 2 compared to cluster 1 (Table 3). These results clearly indicate that production of these cytokines/chemokines after cDC stimulation with Poly:IC decreased with increasing age.

DISCUSSION

We evaluated radiation- and age-associated changes in peripheral blood DCs in a cohort of elderly Japanese A-bomb survivors using a cross-sectional study design. The current study was designed with the hypothesis that radiation exposure would induce impairments of the number and function of DCs, potentially in association with aging-induced immunity attrition. However, we failed to reject the null hypothesis for overall DC counts, cDC counts or pDC, because there were no overall associations with radiation dose. We did observe an overall reduction of DC counts with older ages, and this was also true in the cDC and pDC populations. In terms of function of DCs, we observed no overall change in DC expression levels in association with radiation exposure.

In a previously reported study, we developed assays for the enumeration and characterization of human circulating cDC/pDC precursors and found that there was a strong linkage between DC and T-cell commitments in hematopoietic progenitor cells (*28*). In autopsy thymus specimens from A-bomb survivors, we also found that radiation accelerated human thymic aging and reduced T-cell production in the thymus (*29*), which is concordant with our previously published observations of radiation-associated reductions in peripheral blood naïve T-cell populations among A-bomb survivors (*2*). It is unclear why the data did not support our hypotheses in light of these earlier findings. It is possible that statistical power was insufficient due to the lack of preliminary and/or precedent data in human blood samples, or due to potential biases related to data collected from survivors with relatively healthy aging to more than 65 years old. Data obtained from the current study are based on numerous end points for DC-mediated immunity and are linkable to other immunological and clinical data from the survivors. This may allow us in the future to design a longitudinal study with in-depth analyses of accumulating immunological data, which would have higher power to detect these changes.

In addition to the primary hypothesis, we also performed several additional analyses. We observed dose- and age-dependent declines in the number of circulating DCs among women but not men, and radiation dose was associated with numerical decrement in pDCs but not cDCs. Although there are some inconsistencies with previously reported studies, the age effects on both cDCs and pDCs in the current study population, which is considerably skewed to elderly (the age range of 66 to 91), are somewhat similar in that there are age-dependent decreases in both cDC and pDC populations (*9–14*), and age-associated reductions in cytokine production and TLR expression levels in basal and TLR-stimulated DCs (*9, 10, 12, 13, 15, 16*), in part supporting the hypothesis that human DCs continuously senesce over the lifespan (*30*). On the other hand, Panda *et al*. reported that basal levels of TLR3, TLR7 and TLR8 gene expression in DCs decreased with age (*13*), whereas no age effect on expression of any TLR genes was observed in the current study (data not shown). Age-dependent functional declines observed in the current study involved gene expression profiles related to cDC maturation including pro-inflammatory cytokine and chemokine production in response to Poly I:C stimulation. This observation also conflicts with the previously reported studies on increased pro-inflammatory cytokine production in aged humans, i.e., aging-related elevations in inflammatory cytokine expression in TLR-unstimulated and TLR-stimulated DCs (*13, 31*).

While not fully supporting our hypothesis, we did observe radiation- and age-associated declines in DC numbers in women but not men. Differences in the immune responses between women and men are well known. In particular, responsiveness to viral infection and risks of selected autoimmune diseases are known to be higher in women than in men (*32*). Orsini *et al*. reported the sex difference in DC numbers, which we also found with the same tendency, i.e., greater in men than in women (*14*). Many sex-related

factors are known as biological modifiers for the immune system. In particular, sex hormones strongly affect differentiation of estrogen receptor (ER)-positive lymphoid tissue cells, including lymphocytes and DCs. For example, drastic change of blood estradiol levels in postmenopausal women substantially decreases the numbers of circulating ERα-positive lymphocytes and DCs (*33, 34*). Such effects of hormonal change may be attributable to our current findings of stronger aging effects in women than in men on DCs and other immune cell populations. On the other hand, it has been reported that female-derived pDCs could produce more IFN-γ through TLR7 stimulation than male-derived pDCs (*35, 36*), which was explained by TLR7 gene escaping X chromosome inactivation in women (*37*). Such sex differences may be implicated in the vulnerability of women compared to men to viral infections and autoimmune diseases, such as HIV infection, systemic lupus erythematous and rheumatoid arthritis (*38*). In the current study, however, TLR gene expression was not affected by age or radiation dose, and there was no significant sex difference in IFNA1 gene expression on TLR7-stimulated pDCs. It is speculated that TLR7 expression may decrease in aged female-derived pDCs without affecting IFNA1 expression. In a mouse model study, DC changes in association with radiation exposure were observed in female mice but not in male mice.[2]

Our cluster analysis of gene expression profiles in cDCs identified two clusters of participants that differed in expression levels after TLR stimulation and that are significantly associated with age and chemokine/cytokine production levels. Although the effect of radiation exposure on cDC functions was not apparent, the current study data might provide a molecular epidemiological basis for predicting successful or maladaptive aging of cDC-mediated immunity in elderly men and women. In the future, this could be accomplished with a follow-up study of immunological alterations and disease onsets in this study population. Taken together, the data from this work provide possible clues for radiation-and aging-associated DC alterations that may be at least in part involved in immunity attenuation and host protection failure in elderly A-bomb survivors.

SUPPLEMENTARY INFORMATION

Table S1. List of exclusion criteria.

Table S2. Characteristics of study participants.

Fig. S1. CONSORT diagram of study.

Fig. S2. Dendrogram identifying two major clusters of differentially expressed genes in cDCs. Seventy-eight TLR signaling pathway genes whose expression change in cDCs with Poly I:C stimulation are Z-score normalized to hierarchical clustering. Two major clusters are identified (panel A). One cluster (red) included 45 genes showed differential levels of gene expression in participants (panel B).

Fig. S3. Dendrogram in pDCs. Dendrogram for identifying major clusters of differentially expressed genes in pDCs. Expression changes for 83 TLR signaling pathway genes with R848 stimulation were Z-score normalized and analyzed using hierarchical clustering. The clustering procedure failed to identify clusters of differentially expressed genes.

[2] Personal communication, K. Yasutomo, Tokushima University, Tokushima, Japan.

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