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Kinetics of cytokine mRNA and protein expression by plastic adherent cells in the thymus after split-dose irradiation

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ABSTRACT

Whole body irradiation causes significant apoptosis in various tissues such as the thymus. If apoptotic cells outnumber the phagocytic capacity of macrophages, apoptosis becomes secondary necrosis, inducing inflammatory cytokine expression in macrophages. Radiation also induces thymic lymphomas in C57BL/6 mice after four consecutive irradiations with 1.6 Gy X-rays with nearly 100% incidence. Since cancer development is modulated by a microenvironment involving macrophages, we examined the kinetics of thymocyte number and plastic adherent cell number in the thymus as well as cytokine mRNA expression by plastic adherent cells in the thymus after split-dose irradiation. Upon split-dose irradiation, thymocyte number changed dramatically, whereas plastic adherent cell number did not. Among cytokine mRNAs tested, IL-1β, IL-11 and IL-12p40 mRNAs were up regulated 2 days after the 1st and 2nd, 3rd and 4th, and 2nd and 3rd irradiations, respectively. On the other hand, TNF-α mRNA was up regulated 2 days after the 3rd irradiation and 2 weeks after the 4th irradiation. The level of IL-11 protein was also increased 2 days after 3rd and 4th irradiations. These results suggest that, upon split-dose irradiation, macrophages in the thymus produce various cytokines in a time-dependent manner, thereby contributing to induction of thymic lymphomas.

1. Introduction

Apoptotic and necrotic cells differ in terms of induction of cytokine production upon coculturing with macrophages, the former inducing anti-inflammatory cytokine or mediator production and the latter inducing inflammatory cytokine production [1,2]. When apoptotic cells outnumber the phagocytic capacity of macrophages, for instance, an exposure of mice to whole body irradiation with 1 Gy, apoptosis becomes secondary necrosis, which causes inflammatory cytokine expression in macrophages and subsequent neutrophil infiltration, as demonstrated by us and others [3–5].

Radiation induces thymic lymphomas in C57BL/6 and other strains of mice after four consecutive irradiations with 1.6 Gy X-rays at 4- to 8-day intervals with nearly 100% incidence [6,7], whereas single irradiation with 1.6 Gy X rays does not induce any thymic lymphomas. Many researchers including our group have been exploring the mechanism underlying lymphomagenesis induced by such split-dose irradiation [7–10]. These studies revealed that such split-dose irradiation-induced lymphomagenesis comprises two phases, the first phase occurring 1–4 weeks after the last (4th) irradiation (appearance of prelymphoma cells) and the second one occurring 12 weeks after the last irradiation (growth of lymphoma cells), and that IL-9 receptor α chain mRNA expression increases abruptly prior to the onset of lymphomas in this model.

While cancer development is modulated by a microenvironment involving macrophages, there have been few studies focusing on macrophages in this model. We, therefore, examined the kinetics of the plastic adherent cell number in the thymus as well as that of cytokine mRNA and protein expression by plastic adherent cells in the thymus after split-dose irradiation, comparing the results with the kinetics of thymocyte number to gain an insight into the involvement of plastic adherent cell-derived cytokines in lymphomagenesis.

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2. Materials and methods

2.1. Mice and irradiation

Female C57BL/6J mice were produced by the National Institute of Radiological Sciences (NIRS), National Institutes for Quantum and Radiological Science and Technology (QST) of Japan, and housed in a temperature- and light-controlled facility with food and water available ad libitum. Animal care and experimental schedules were approved by the Institute and were in strict accordance with the guidelines of the Institute. Five-week old female mice were exposed to whole-body irradiation (1.6 Gy per exposure at 0.5 Gy/min) once a week for four consecutive weeks in a perforated Plexiglas apparatus using an X-ray-generator (Pantak Ltd., East Haven, CT; 200 kVp, 20 mA with filters of 0.5 mm Cu and 0.5 mm Al). Age-matched mice not irradiated were used as controls.

2.2. Preparation of thymocytes and plastic adherent cells

Mice were sacrificed by cervical dislocation 2 days and 1 week after each irradiation (just before next irradiation). Irradiated mice were also sacrificed 2 and 4 weeks after the 4th irradiation, as shown in Fig. 1 (black circles). Control mice were sacrificed at 5, 8, 9, 10 and 12 weeks after birth, as also shown in Fig. 1 (white circles). Then their thymuses were surgically removed and weighed.

To isolate thymocytes, the thymuses were squeezed between slide glasses, and cells were suspended in ice-cold phosphate-buffered saline (PBS) and filtered twice through a nylon mesh.

To isolate plastic adherent cells, five thymuses were pooled, cut into small pieces with scissors, and then incubated with 3 ml collagenase N-2 (133 U/ml) (Niita Gelatin, Tokyo) at 37 °C for 30 min, followed by vigorous pipetting and filtration through a cell strainer (Falcon, Tokyo). Cells were then layered over FCS, followed by centrifugation and adherence onto plastic dishes at 37 °C for 2 h.

2.3. Identification of macrophages and assaying for phagocytosis

To identify macrophages, plastic adherent cells were washed with PBS, fixed with methanol and then incubated with Block Ace (Dainippon Pharmaceutical Co., Osaka) containing 10% normal goat serum at room temperature for 1 h. They were then incubated with FITC-labeled rat anti mouse F4/80 antibodies (Bio-Rad, Kidlington, UK) containing 1% normal goat serum at 4 °C for 1 h. They were observed under a fluorescence microscope (IX71, Olympus, Tokyo) after adding VECTASHIELD (Vector, Burlingame, CA) to the cells.

As for phagocytosis assaying, plastic adherent cells were washed with RPMI1640 medium containing 10% FCS (medium), and then incubated with 0.00625% carboxylate-modified latex beads (FluoSpheres, 1 μm; Molecular Probes, Eugene OR) at 37 °C for 1 h. Of note is that carboxylate-modified latex beads are phagocytosed by macrophages in a similar way to apoptotic cells [11]. Cells were then washed with medium and PBS, stained with anti F4/80 antibodies as described above, fixed with methanol and observed under a fluorescence microscope.

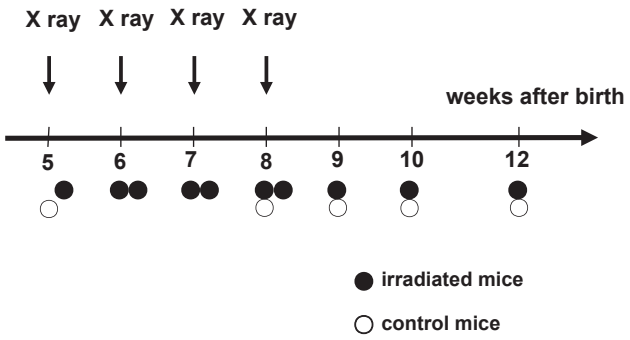

Fig. 1. Experimental design.

2.4. Quantitative reverse transcription-PCR (qRT-PCR)

Plastic adherent cells were lysed with a 4 M guanidine thiocyanate solution (Sigma) containing 0.5% sodium lauroyl sarcosinate and 25 mM citrate buffer (pH 7), and stored at -80 °C until assaying. Total RNA was then extracted with guanidine thiocyanate and phenol. First-strand cDNA was synthesized from total RNA and used for qRT-PCR. Using SYBR Green Master Mix (Applied Biosystems, Foster City, CA), the expression of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, as an internal standard, and the genes of interest was measured by real time PCR (ABI PRISM 700). The dissociation curve was confirmed for each primer, and any cases involving anomalous gene amplifications were excluded from the analysis. The threshold cycles (Ct) for genes of interest and GAPGH were determined and the expression levels were calculated by the ΔΔCt method. The PCR amplification program consisted of denaturation at 95 °C for 10 sec, and 45 subsequent amplification cycles of denaturation at 95 °C for 15 sec and annealing/elongation at 60 °C for 1 min. The primer sequences were as follows; GAPDH forward tcgtcccgtagacaaaatgg, GAPDH reverse tctcggccttgactgtgc, IL-1β forward gaccttccaggatgaggaca, IL-1β reverse tccattgaggtggagagctt, IL-11 forward tgacggagatcacagtctgg, IL-11 reverse ggagtagccgttccagtcg, IL-12p40 forward agcagtagcagttcccctga, IL-12p40 reverse agtccctttggtccagtgtg, IL-6 forward gttgtgcaatggcaattctg, IL-6 reverse ctctgaaggactctggctttg, TNF-α forward gcctcttctcattcctgctt, and TNF-α reverse cacttggtggtttgctacga.

2.5. Measurement of cytokine in plasma and plastic adherent cell culture supernatants

Plasma samples were obtained by centrifugation of blood samples at 1500*g* for 10 min and stored at −30 °C until use. Plastic adherent cells were cultured in 24 well plates at a cell density of 5×10^4 cells/ml for 24 h. Then culture supernatants were obtained by centrifugation at 1500*g* for 10 min and stored at −30 °C until use. IL-1β, IL-11 and IL-12p40 levels were measured with Luminex xMAP kits (Mouse Cytokine/Chemokine Magnetic Beads panel I for IL-1β and IL-12p40, panel II for IL-11, Luminex Co., Austin, TX, USA). The detection limits of IL-1β, IL-11 and IL-12p40 levels were 3.2 pg/ml, 12.2 pg/ml and 3.2 pg/ml, respectively. Each sample was measured in duplicate, and the mean value was used for statistical analysis.

2.6. Statistical analysis

Statistical analysis of data on thymocyte number, plastic adherent cell number, mRNA and protein levels was performed by means of one-way ANOVA followed by Tukey's test. The chosen level of significance was $p < 0.05$.

3. Results

3.1. Changes in thymocyte number and plastic adherent cell number after split-dose irradiation

We determined the thymocyte number and plastic adherent cell number for the thymuses of normal and irradiated mice after split-dose irradiation (Fig. 2A and B). In normal mice, the thymocyte number peaked at 8 weeks after birth, and thereafter returned to that at 5 weeks after birth. In irradiated mice, on the other hand, at 2 days after irradiation the number was decreased to one tenth of that before irradiation and returned to that before irradiation 1 week after the 1st, 2nd and 3rd irradiations ($p < 0.01$). After the 4th irradiation, on the contrary, the number did not return to that before irradiation even after

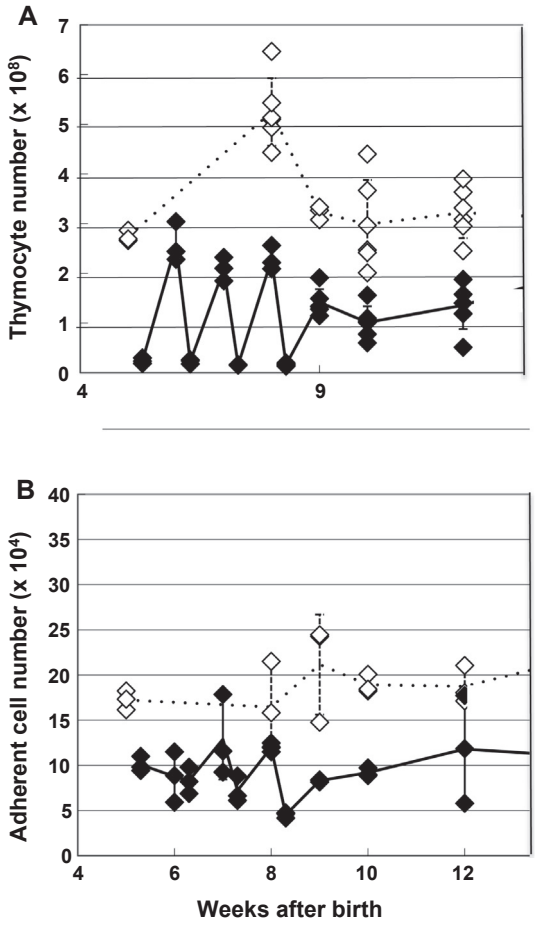

Fig. 2. Changes in thymocyte number and adherent cell number after split-dose irradiation. (A) Mice were exposed to whole-body irradiation (1.6 Gy per exposure at 0.5 Gy/min) once a week for 4 consecutive weeks. The thymocyte numbers in irradiated and control mouse were then determined at the indicated times. The thymocyte number in an irradiated mouse is shown as a black diamond, whereas that in a control mouse is shown as a white diamond. The data are also expressed as the means ± standard error for 3 mice. (B) The plastic adherent cell numbers in thymuses of irradiated and control mice were determined at the indicated times. The cell number in the thymus of an irradiated mouse is shown as a black diamond, whereas that in a control mouse is shown as a white diamond. The data are also expressed as the means ± standard error for 3 mice.

4 weeks, although statistically not significant.

Changes in the plastic adherent cell number were different from those in the thymocyte number in terms of time kinetics (Fig. 2B). Although the changes in the plastic adherent cell number in normal and irradiated mice were not statistically significant, there were some trends in irradiated mice. At 2 days after the 1st irradiation the number was decreased to one half of that before irradiation, but did not return to that before irradiation 1 week later. At two days after the 2nd irradiation, the number was not decreased, as compared with the number before irradiation. The number then was increased 1 week after the 2nd irradiation. At two days after the 3rd irradiation, the number was decreased as compared with the number before irradiation and return to that before irradiation 1 week after the 3rd irradiation. At two days after the 4th irradiation, the number was decreased as compared with the number before irradiation, but did not return to that before irradiation until 4 weeks after the 4th irradiation.

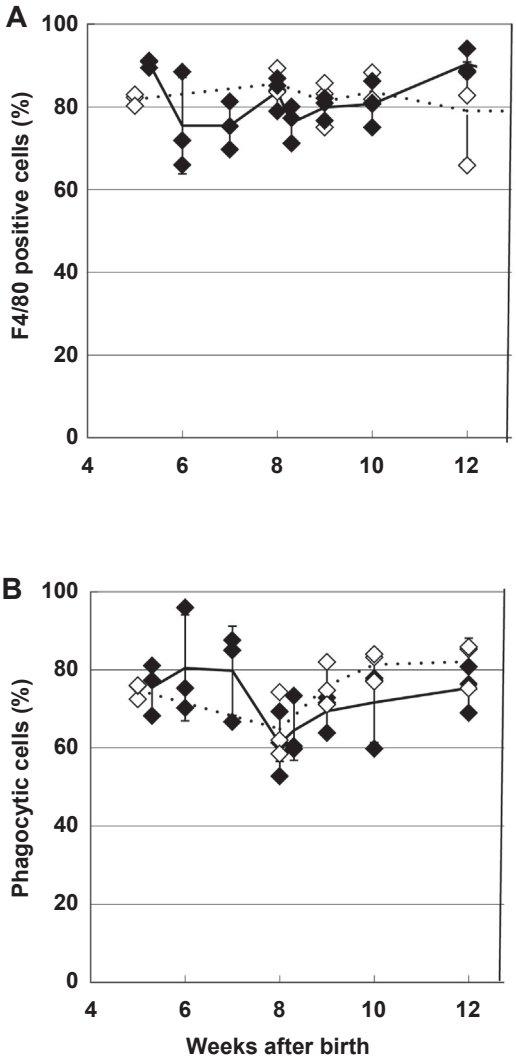

Fig. 3. Changes in F4/80 positive cells and phagocytic cells among plastic adherent cells after split-dose irradiation. (A) Mice were exposed to whole-body irradiation (1.6 Gy per exposure at 0.5 Gy/min) once a week for 4 consecutive weeks. Plastic adherent cells were then obtained at the indicated times (5.3 wks, 6 wks, 7 wks, 8 wks, 8.3 wks, 9 wks, 10 wks, 12 wks after birth). Cells of control thymuses were also obtained (5 wks, 8 wks, 9 wks, 10 wks, 12 wks after birth). The percentage of F4/80 positive cells from an irradiated mouse was then determined and is shown as a black diamond, whereas that from a control mouse is shown as a white diamond. The data are also expressed as the means ± standard error for 3 mice. (B) The percentage of cells phagocytosing latex beads from an irradiated mouse was determined and shown as a black diamond, whereas that from a control mouse is shown as a white diamond. The data are also expressed as the means ± standard error for 3 mice.

3.2. Changes in F4/80-positive cells among plastic adherent cells and phagocytic activity

We then determined the percentages of F4/80-positive cells among plastic adherent cells and phagocytic activity toward carboxylate-modified latex beads.

Plastic adherent cells from thymuses of control and irradiated mice comprised approximately 77 and 72% of F4/80-positive cells, respectively. There were no significant differences in the percentages of F4/80-positive cells between control and irradiated mice at the same age (Fig. 3A).

Plastic adherent cells from the thymuses of control and irradiated mice comprised approximately 77 and 72% of cells phagocytosing latex beads, respectively. There were no significant differences in the

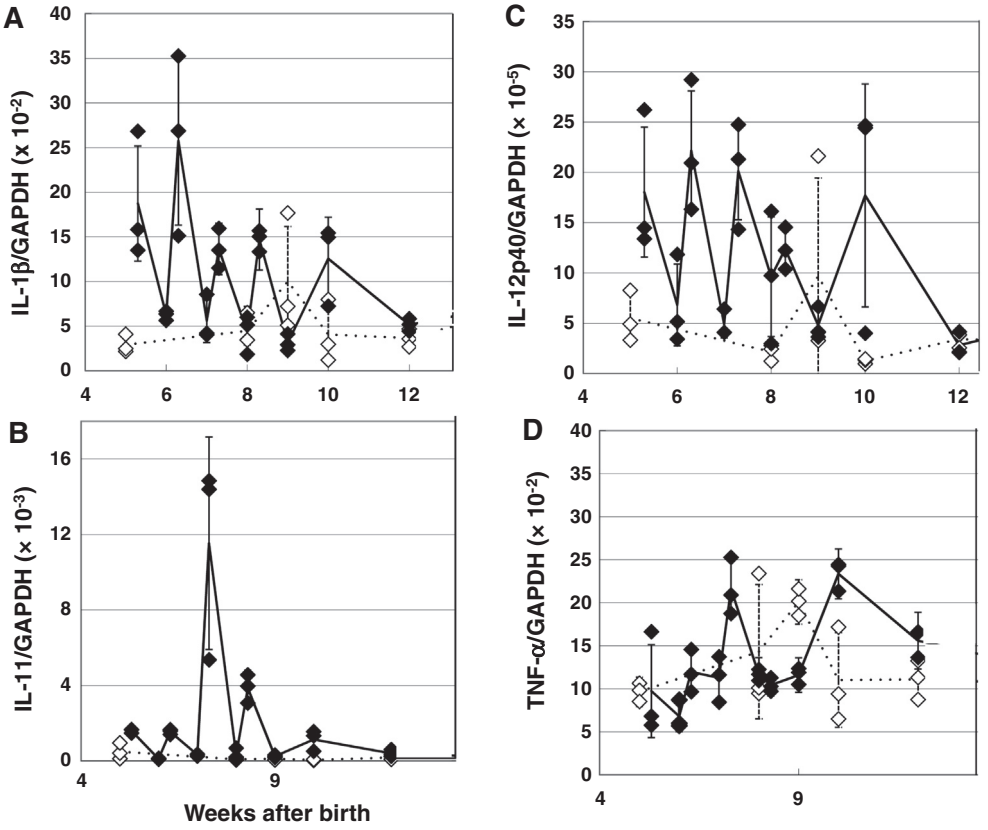

Fig. 4. Changes in IL-1β, IL-11, IL-12p40 and TNF-α mRNA expression in plastic adherent cells. The IL-1β (A), IL-11 (B), IL-12p40 (C) and TNF-α (D) mRNA levels relative to those of GAPDH mRNA were determined with qPCR as described under Materials and Methods. The data are expressed as the means ± standard error for 3 groups of mice, each group comprising 5 mice. The data for irradiated mice are shown as black diamonds, whereas those for control mice are shown as white diamonds.

percentages of phagocytosing cells between control and irradiated mice at the same age (Fig. 3B).

3.3. Changes in cytokine mRNA expression in plastic adherent cells after split-dose irradiation

We then examined the kinetics of expression of IL-1β, IL-11, IL-12p40 and TNF-α mRNAs by plastic adherent cells after split-dose irradiation. These cytokines were chosen because all of them are reportedly involved in regulation of T cell growth or differentiation either directly or indirectly [12–16].

The expression of IL-1β, IL-11, IL-12p40 and TNF-α mRNAs changed with characteristic patterns of kinetics, as shown in Fig. 4A to D. IL-1β mRNA expression was increased 2 days after the 1st and 2nd irradiations as compared with that before each irradiation ($p < 0.01$, Fig. 4A). IL-11 mRNA expression was increased 2 days after the 3rd and 4th irradiations by 130- and 45-fold as compared with that before each irradiation, respectively ($p < 0.01$, Fig. 4B). IL-12p40 mRNA expression was increased 2 days after the 2nd and 3rd irradiations as compared with that before each irradiation ($p < 0.05$, Fig. 4C). TNF-α mRNA expression was increased 2 days after the 3rd irradiation and 2 weeks after the 4th irradiation as compared with that before each irradiation ($p < 0.01$, Fig. 4D).

3.4. Changes in cytokine protein levels in plasma and plastic adherent cell culture supernatants after split-dose irradiation

We then determined the protein levels of IL-1β, IL-11 and IL-12p40 in plasma and plastic adherent cell culture supernatants. Although IL-1β and IL-12p40 were detected in both samples, changes were not statistically significant (Fig. 5A and B). On the other hand, changes in IL-11 in plastic adherent cell culture supernatants were statistically significant ($p < 0.05$, no irradiation vs. 7.3 wks; $p < 0.05$, 5.3 wks vs. 8.3 wks; $p < 0.01$, no irradiation vs. 8.3 wks) (Fig. 5), whereas changes in IL-11 in plasma were not (Fig. 5C).

4. Discussion

After split-dose irradiation, thymocyte number changed dramatically. Two days after each irradiation, it was decreased to one tenth of the number before irradiation, whereas, one week after each irradiation, it returned to the levels before irradiation except for the 4th irradiation. This is because $CD4^-8^-$ thymocytes as well as proT cells from bone marrow proliferate after the 1st, 2nd and 3rd irradiations [10], and because T precursor cells are not supplied by bone marrow after the 4th irradiation [17]. In contrast, the plastic adherent cell number changed differently. Although the exact reason for differences in the changes of thymocyte and plastic adherent cell numbers is not known at present, it is perhaps because thymocytes and thymic macrophages differ in terms of sensitivity toward irradiation [18]. There is also the possibility that thymocytes and thymic macrophages are restored after irradiation through different mechanisms, namely proliferation of radioresistant thymocytes and infiltration of monocytes, respectively. It is of note that exposure of mice to whole body irradiation causes apoptosis and secondary necrosis of thymocytes, the latter inducing inflammatory cytokine expression in macrophages and subsequent neutrophil infiltration in the thymus [3–5], followed by recruitment of monocytes and their differentiation into macrophages [19].

There were no significant differences in the percentages of phagocytic cells and those of F4/80-positive cells among plastic adherent cells between control and irradiated mice at the same age. This is contrary to our expectation that the phagocytic activity of macrophages may differ 2 days and 1 week after irradiation because many dead cells would be phagocytosed by macrophages during the 2 days after irradiation, whereas fewer dead cells would be phagocytosed after that. The results indicate that the phagocytic activity of macrophages is less affected by the number of dead cells.

When we examined cytokine mRNA expression in plastic adherent

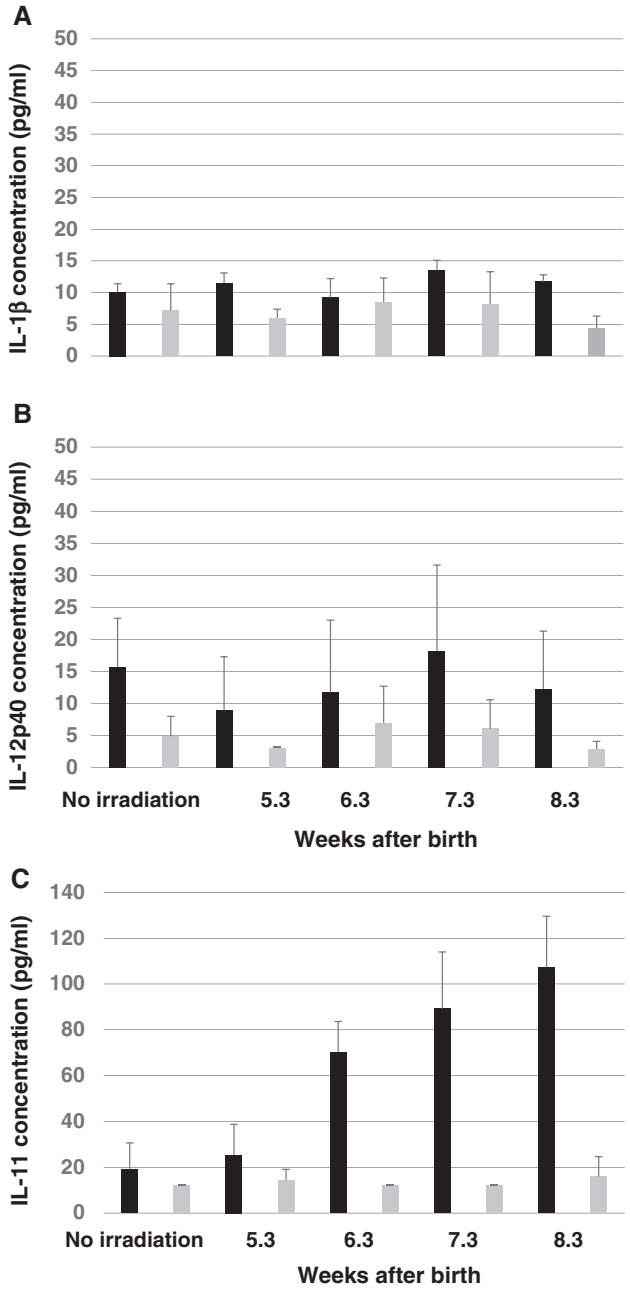

Fig. 5. Changes in IL-1β, IL-12p40 and IL-11 protein levels in plastic adherent cell culture supernatants and plasma. Mice were exposed to whole-body irradiation (1.6 Gy per exposure at 0.5 Gy/min) once a week for 4 consecutive weeks. Plastic adherent cells were then obtained at the indicated times (5.3 wks, 6.3 wks, 7.3 wks, 8.3 wks after birth). Cells of control thymuses were also obtained 5 wks after birth. Plasma were also taken from individual mouse. IL-1β (A), IL-12p40 (B) and IL-11 protein levels (C) in plastic adherent cell culture supernatants (black) and plasma (grey) were determined as described under Materials and Methods. The data are expressed as the means ± standard error for 3 wells, which were prepared from 5 mice, for plastic adherent cell culture supernatants or as the means ± standard error for 5 mice for plasma.

cells after split-dose irradiation, we found different patterns of kinetics. The IL-1β, IL-11 and IL-12p40 mRNAs were up regulated 2 days after the 1st and 2nd, 3rd and 4th, and 2nd and 3rd irradiations, respectively. Of note was that IL-11 mRNA expression was increased very greatly 2 days after the 3rd and 4th irradiations by 130- and 45-fold as compared with the control, respectively. On the contrary, TNF-α mRNA was up regulated 2 days after the 3rd irradiation and 2 weeks after the 4th irradiation.

IL-1β, IL-11 and IL-12p40 were detected in plasma and plastic adherent cell culture supernatants. However, changes in IL-1β and IL-12p40 protein levels were not statistically significant. This is probably because activation of IL-1β processing enzyme may have occurred insufficiently and because there were only 5-fold increases in mRNA levels of IL-1β and IL-12p40 as compared with that in IL-11 mRNA. On the other hand, changes in IL-11 protein levels in culture supernatants were statistically significant 2 days after the 3rd and 4th irradiations, this being in good agreement with the data of RT-PCR. Changes in plasma IL-11 levels, however, were not statistically significant, suggesting either that IL-11 acts locally in the thymus and/or that IL-11 has a short half-life in the blood. The former possibility is more likely than the latter one, because leukemogenesis occurs primarily in the thymus under the condition we employed, although the latter possibility cannot be excluded.

IL-1 is involved in early T- cell expansion and differentiation, because CD4lo cells were found to proliferate best in response to the combination of IL-1, IL-3, IL-6, IL-7, and stem cell factor [12]. Although there has been a report that irradiation induced IL-1β mRNA expression in macrophages directly, the expression had returned to the basal level after 4 h [20]. Consequently, it is likely that IL-1β mRNA is produced through the interaction of macrophages with secondary necrotic cells in the thymus, as reported previously [21].

IL-11 is a member of the IL-6 family with potent thrombopoietic activity, and it reportedly suppresses irradiation-induced TNF-α production [13], suggesting the possibility that IL-11 supports the proliferation of prelymphoma cells by suppressing TNF-α-induced apoptosis. This possibility may be supported by that prelymphoma cells appear 1–4 weeks after the 4th irradiation [8]. Such a large increase in IL-11 mRNA 2 days after the 3rd and 4th irradiations may also indicate an unknown role in leukemogenesis, such as as-yet unproven recruitment of hematopoietic progenitor cells to the thymus, via thrombopoietin-mediated proliferation of hematopoietic stem cells [14].

IL-12p40, on the other hand, sends positive signals for intrathymic T cell development [15]. Since we previously reported that IL-12p40 was produced on coculturing macrophages with secondary necrotic cells [22], it is likely that IL-12p40 is produced by macrophages interacting with secondary necrotic cells in the thymus.

Since TNF-α is expressed at the time when prelymphoma cells begin to appear, TNF-α presumably suppresses the appearance of prelymphoma cells through induction of apoptosis. In support of this, Humblet et al. reported that intraperitoneal injection of TNF-α inhibits the development of thymic lymphomas [23], although they did not examine whether or not TNFα is produced in the thymus after split-dose irradiation to prevent leukemogenesis.

IL-6 mRNA was not detected in our time schedule (data not shown). There has been a report, however, that IL-6 was produced in the thymus after irradiation and was involved in acute regrowth of the thymus after genotoxic stresses [24]. In this study, the majority of IL-6 was secreted from thymic endothelial cells, whereas resident macrophages produced IL-6 at a level that was more than ten-fold less than that produced by endothelial cells. Determination of whether or not another microenvironment than macrophages contributes to lymphomagenesis through production of cytokines such as IL-11 and TNF-α awaits further investigation.

Why are cytokines produced with different time kinetics upon split-dose irradiation? Although the exact reason is not known at present, it could be due to genetic alteration of macrophages and changes in damage-associated molecular patterns released from primary or secondary necrotic cells. Interaction of macrophages with surrounding cells would also contribute to the difference in time kinetics of cytokine production. Many more studies are required to answer this question.

Finally, thymuses that weighed over 100 mg appeared at 4 weeks after the 4th irradiation and the number of such thymuses was increased in a time-dependent manner thereafter (data not shown),

suggesting that thymic lymphomas were induced in this model.

In conclusion, our study revealed that, upon split-dose irradiation, the thymocyte number, plastic adherent cell number and cytokine mRNA levels after each irradiation were not merely repetitions of those after a single irradiation, as similar changes in thymus weight were previously reported [10]. Our study also raised the possibility that macrophages may play a critical role in lymphomagenesis through production of cytokines such as IL-11 and TNF-α at specific stages. This possibility is worthy further investigation.

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Declarations of interest

None.

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