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*Regular Paper*

# Effects of Low-dose Irradiation on Enhancement of Immunity by Dendritic Cells

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**Low-dose Irradiation/Dendritic cell/T cell activation/Th1.**

Low-doses of irradiation have been reported to have beneficial effects, particularly anti-tumor effects. In this paper, we show the effects of the low-dose irradiation on T cell activation induced by dendritic cells (DCs). DCs, which had been pre-irradiated at 0.02–1.0 Gy from a $^{137}$Cs source, were cultured with allogeneic T cells, and the proliferation of T cells was then examined. The 0.05Gy-pre-irradiated DCs showed the highest proliferation capacity of T cells. The 0.05Gy-irradiation does not augment the expression of major histocompatibility complexes (MHCs) or costimulatory molecules on DCs, as with non-irradiated DCs or 1Gy-irradiated DCs, but does augment the production of IL-2, IL-12 and IFN-γ DCs. These results suggest that the low-dose irradiation augments T cell-activation capacity through cytokine production by DCs, which might shift naïve helper T cells to Th1 cells.

## INTRODUCTION

Dendritic cells (DCs), which differentiate from hematopoietic stem cells in the BM, are well known to have the strong capacity to present antigens to T cells and induce the activation of T cells.[1)] Although DCs reside in all organs, their numbers are very small.[2)] However, DCs play a central role in the orchestration of the various forms of immunity and tolerance.[3)]

It has been stated that low-dose irradiation has stimulating effects on not only plant growth but also the proliferation of mammalian cells.[4–6)] It has also been reported that low-dose irradiation might activate the immunological network[7)] and have some anti-tumor effects. Experimentally, low-dose irradiation can suppress the metastasis of cancer cells[8)] and the generation of cancers.[9,10)] In epidemiological studies, atomic bomb survivors who had been exposed to low-level irradiation have been reported to have significantly lower mortality from cancerous diseases.[11)] Hashimoto *et al.* have reported that low-doses of irradiation histologically accelerate the infiltration of lymphocytes into tumors, resulting in the suppression of metastasis of the tumor.[8)] It has also been reported that low-dose irradiation augments the activity of T cells, NK cells and B cells.[12,13)] Kojima *et al.* have also reported that low-dose irradiation induces the proliferation of spleen cells, and that it shows anti-tumor effects via the induction of glutathione.[14,15)] However, the role of DCs in low-dose irradiation has not been clarified.

In this paper, we show that the low-dose irradiation given to DCs augments the stimulatory activity of the DCs on allogeneic T cells, resulting from augmented production of IL-2, IL-12 and IFN-γ.

## MATERIALS AND METHODS

### *Mice*

Eight-week-old male C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC inc. (Shizuoka, Japan). All animal use was conducted in accredited facilities and was approved by the Animal Care Committee of Kansai Medical University.

### *Isolation of mononuclear cells from spleens*

To obtain single-cell suspensions from the spleens of mice, we injected PBS containing 150 U/ml collagenase into the spleens. The spleens were cut into small pieces, and then digested in the PBS with collagenase. These cells were

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resuspended in PBS.

*Irradiation*

Splenic DCs from B6 mice were exposed to various irradiation doses (0.02, 0.05, 0.1, 0.5, or 1 Gy at 1.0 Gy/min) from a $^{137}$Cs source (Gammacell 40 Exactor; MDS Nordion International Inc., Ottawa, Ontario, Canada). Irradiated cells were incubated for indicated periods for further studies, as described below.

*Reagents*

Phycoerythrin (PE)-labeled anti-CD11c, fluorescein isothiocyanate (FITC)-labeled anti-IA$^b$, FITC-labeled anti-B7.1 (CD80), FITC-labeled anti-B7.2 (CD86), FITC-labeled anti-ICAM-1, FITC-labeled CD11a (LFA-1), FITC-labeled anti-CD1d, biotin-labeled anti-CD19 antibodies (Abs), isotype matched control Abs and Via-Probe (cell viability solution) were purchased from BD Biosciences (San Jose, CA, USA). Biotin-labeled anti-CD3 and allophycocyanin (APC)-labeled anti-B220 Abs were from Caltag (Burlingame, CA, USA), and FITC-labeled anti-CD40 Ab was from Sumitomo Electric Industries, Ltd (Osaka, Japan). FITC-labeled anti-rat IgG Ab was from Biosource International (Camarillo, CA, USA). Red PE-Cy5-labeled streptavidin was from DakoCytomation Japan (Kyoto, Japan). Collagenase was from Sigma Chemical (St. Louis, MO, USA). MACS magnetic microbeads and the column for cell separation were from Miltenyi Biotec (Bergisch Gladbach, Germany).

*Enrichment of DCs and T cells from spleen*

For the enrichment of the splenic DCs from the B6 mice, we used anti-CD11c Ab-coupled MACS magnetic beads and the column for MACS magnetic beads. Isolated spleen cells were incubated with anti-CD11c Ab-coupled MACS, followed by passing through a MACS cell sorter (Milteneyi Biotec) for positive selection of CD11c$^+$ cells. Thus obtained cells, which showed more than 80% positive for CD11c, were used as DCs.

To obtain T cells as responders, spleen cells from BALB/c (H2$^d$) mice were incubated with anti-Thy1.2 Ab-coupled MACS magnetic beads, followed by passing through a MACS cell sorter. More than 90% of the thus selected cells were positive for CD3.

*Allogeneic mixed lymphocyte reaction*

After irradiation of DCs at various doses, the indicated stimulator cells (DCs) ($1 \times 10^5$ cells) were cultured in each well of a 96-well flat-bottomed tissue-culture plate (Corning, New York, NY, USA) for 48 hours. After 25Gy irradiation of DCs, T cells ($1 \times 10^5$ cells) were added to the DCs in each well. After incubation at 37°C for another 48 hours in a $CO_2$ incubator, cell proliferation was examined using 2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl) -5-(2,4-disulfophenyl) -2H-tetrazolium, monosodium salt (WST-8; Nakarai, Kyoto, Japan). Twenty μl of WST-8 (10 mM) was added to each well and the plates were incubated at 37°C for an additional 4 hours. The resultant absorbance at 450 nm was read using a microplate reader (Bio-Rad Laboratories; Hercules, CA, USA).

*Cell surface staining*

For flow cytometric analyses, cells were stained with biotin-labeled anti-CD3, biotin-labeled anti-CD19, APC-labeled anti-B220, PE-labeled anti-CD11c and FITC-labeled indicated Abs, followed by staining with Red PE-Cy5-avidin and Via-Probe. The samples were analyzed using a FACScan flow cytometer (Becton Dickinson).

*Reverse transcriptase polymerase chain reaction (RT-PCR) for detection of mRNA expression*

RNA preparation, cDNA synthesis, and PCR were carried out. Total cellular RNA was prepared using a nucleic acid extractor (TRIZOL Reagent, Invitrogen Life Technologies; Carisbad, CA, USA) followed by chloroform extraction and isopropanol precipitation. cDNA was synthesized using RT (M-MLV Rtase in RT-PCR high [RT-PCR Kit], TOYOBO; Tokyo, Japan) and Oligo(dT)$_{20}$- P7 primers (RT-PCR high). PCR was performed on the cDNA using the following primers for G3PDH (RT-PCR high), interleukin-12 (IL-12) primer for p40 subunit, IL-2 IFN-γ, CCR6 and CCR7 (Maxim Biotech, Inc.; San Francisco, CA, USA) with thermal cycling amplification using Takara PCR Thermal Cycler MP (Takara Bio, Otsu, Japan). PCR products were separated on a 1.2% agarose gel (Invitrogen Life Technologies) and visualized by ethidium bromide (Nakarai) staining.

*ELISA assays for supernatant IL-12 and IL-10*

IL-12 and IL-10 levels in culture supernatants of cultured DCs ($1 \times 10^5$/ml) from B6 splenic cells were measured 48 hours after exposure to radiation by using enzyme-linked immunosorbent assay (ELISA) kits (Immunoassay Kit from Biosource International) following the manufacturer's instructions.

*Statistical analyses*

Differences between groups were evaluated using the Student's *t*-test. *p* values of less than 0.05 were considered to be statistically significant.

## RESULTS

*Low-dose-irradiated DCs accelerate proliferation of T cells*

First, we examined the stimulator activity of DCs irradiated with various doses (0Gy, 0.02Gy, 0.05Gy, 0.1Gy, 0.5Gy and 1Gy), using WST-8 (Figs. 1A). The wells in which T cells were cocultured with the DCs irradiated at low doses (0.02, 0.05 and 0.1Gy) showed higher density of WST-8

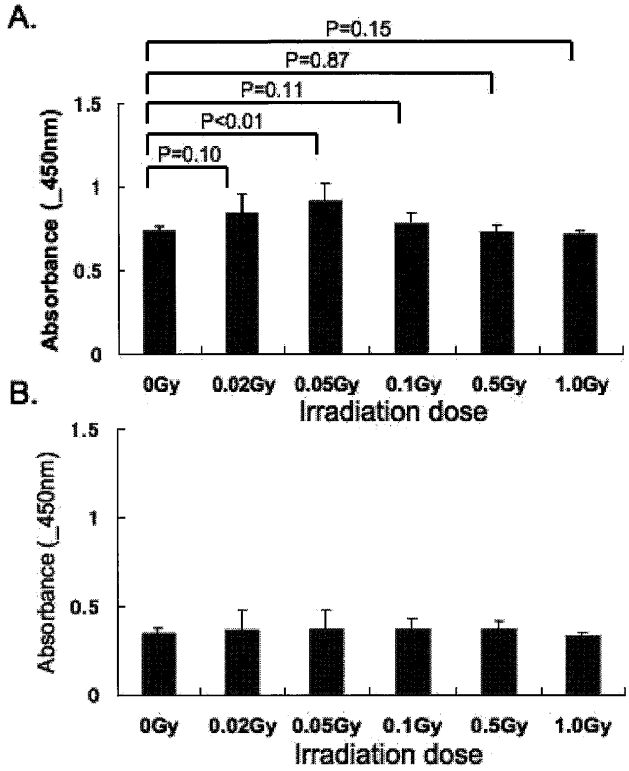


**Fig. 1.** Low-dose irradiation enhances T cell-activation capacity of DCs. A. DCs ($1x10^5$ cells), which had been enriched from spleen cells of B6 using anti-CD11c coated MACS magnetic beads, were irradiated at several low doses. After 2 days of culture, the DCs were irradiated at 25 Gy to inhibit their proliferation. T cells ($1 \times 10^5$ cells) from spleen of BALB/c were added the DCs, and they were cultured for another 2 days, followed by the analyzes of T cell proliferation to examine the T cell activation capacity of DCs using WST-8, as described in the "Materials and Methods". Means and SDs are shown. n.=4. Representative data from 3 independent experiments are shown. B. DCs, which had been enriched from spleen cells of B6 using anti-CD11c coated MACS magnetic beads, were irradiated at several low doses. After 2 days of culture, WST-8 was added to the DCs to analyze cell number of DCs for the examination of the proliferation of DCs. Means and SDs are shown. n.=6. Representative data from 3 independent experiments are shown.

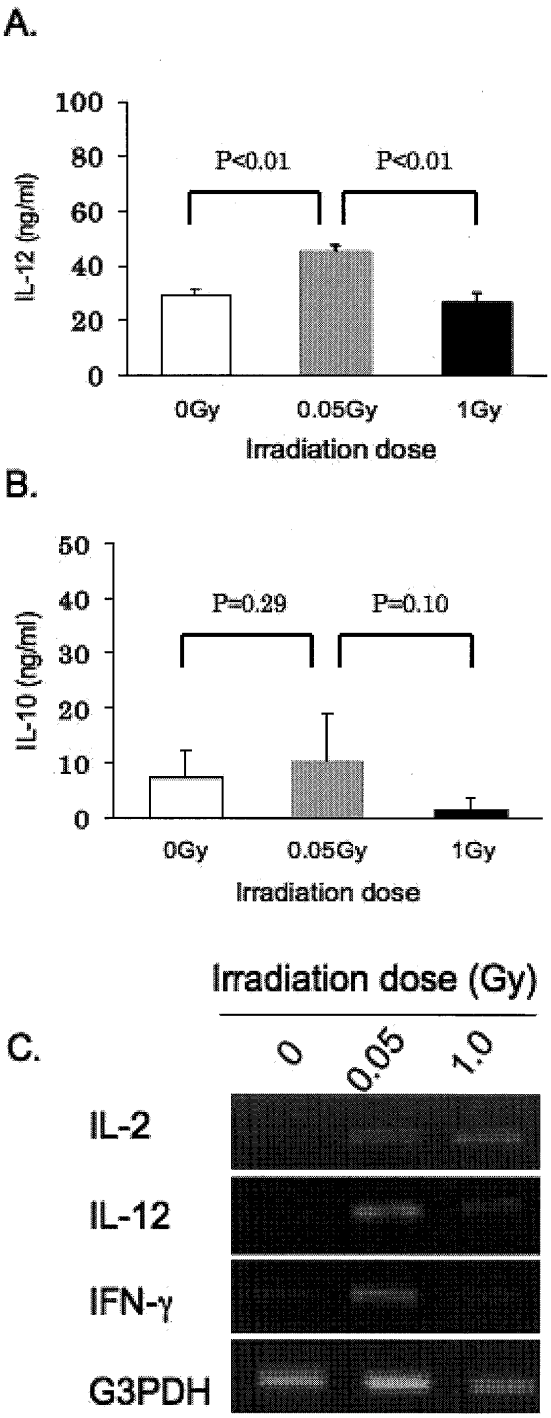


**Fig. 2.** Low-dose irradiation significantly augments the production of IL-12 but not IL-10. DCs ($1 \times 10^5$/ml), which had been irradiated at 0, 0.05 or 1.0 Gy, were cultured for 2 days, and the supernatants were collected and the concentrations of IL-12 (A) and IL-10 (B) were measured using ELISA kit, as described in the "Materials and Methods". Means and SDs are shown in each group. n.=4. Representative data from 2 independent experiments are shown. C. DCs, which had been irradiated at 0, 0.05 or 1.0 Gy, were cultured for 8 hours and then the DCs were collected for extraction of RNA. Next, RT-PCR using the extracted RNA was carried out, as described in the "Materials and Methods". Representative data from 2 independent experiments are shown.

than in non-irradiated DCs or DCs irradiated at higher doses (0.5Gy and 1Gy). In this experiment, we used irradiated DCs at 25 Gy before coculture with T cells to inhibit the proliferation of DCs. Therefore, the augmented cell numbers should reflect an increase in T cell numbers.

Next, we examined the effects of low dose irradiation on the proliferation of DCs. As shown in Fig. 1B, the low-dose irradiation has no effects on the proliferation of DCs. Therefore, the increased cell numbers at the low-dose irradiation were attributable not to the proliferation of DCs but to the proliferation of T cells. The peak dose of irradiation for DC activation is 0.05Gy, while the effects of irradiation decreased gradually in a dose dependent manner. These results suggest that DCs irradiated at low doses accelerated

the proliferation of T cells and the effect cannot be attributed to the proliferation of DCs.

*Low-dose irradiation augments IL-12 production from DCs*

To clarify the mechanisms underlying the augmentation of T cell activation by the low-dose irradiated DCs, we first examined the expressions of activation markers and costimulatory molecules of DCs, such as CD1d, CD40, CD80, CD86, ICAM-1, LFA-1 and MHC class II. It has been reported that DCs present the fragments of antigen peptides through interactions between MHC molecules and T cell receptor (TCR), and that costimulatory molecules (CD40, CD80, CD86 etc.) augment the effects. However, the expression of these antigens remained unchanged in the low-dose irradiated DCs in comparison with non-irradiated DCs. It has also been reported that DCs activate T cells through several cytokines. IL-10 from DCs is notable in that it directs helper T cells toward Th2, while IL-12 from DCs directs helper T cells toward Th1 cells.[23,24] Therefore, we examined the production of IL-10 and IL-12 from the low-dose-irradiated DCs. As shown in Fig. 2 A and B, the supernatants of the low-dose irradiated DCs contain higher concentrations of IL-10 and IL-12. However, there was no significant difference in the concentrations of IL-10 among non-irradiated DCs, the low-dose irradiated DCs and high-dose irradiated DCs (Fig. 2B). The supernatants of the low-dose irradiated DCs contain significantly higher concentrations of IL-12 than those of non-irradiated DCs or high-dose irradiated DCs. Therefore, we further examined the production of Th1 cytokines (IL-12, IL-2 and IFN-γ) using RT-PCR. As shown in Fig. 2C, even 8 hours after irradiation, 0.05Gy-irradiated DCs expressed relatively high mRNA levels of IL-2, IL-12 and IFN-γ. These results suggest that the low-dose irradiation induces Th1 cytokines. We also examined the mRNA expression of CCR6 and CCR7, since it has been reported that immature DCs express CCR6, while mature DCs express CCR7.[3,16,17] However, the mRNA expression of CCR6 or CCR7 remained unchanged after irradiation (data not shown).

## DISCUSSION

Muller HJ reported that X-rays are mutagenic, and that there is a linear relationship between mutation rates and irradiation doses.[18] The International Commission on Radiological Protection (ICRP) also proposes that there is no safe level of irradiation- the so-called linear no threshold (LNT) model. From this theory, damage from irradiation increases in a dose-dependent manner. However, there are many contradictory experimental results and epidemiological research data indicating the beneficial effects of low-dose irradiation.

The low-dose irradiation has been reported to activate T cells, NK cells and B cells, resulting in activation of the immune system and suppression of tumor growth.[12,13,19,20] DCs have been reported to be able to activate not only T cells but also NK cells and B cells, and have also been reported to be important for the regulation of the immune system, like the conductor of an orchestra.[21,22] These facts encouraged us to examine the effects of low-dose irradiation on activation of DCs. Kojima *et al.* have reported that the low-dose irradiation augments the Con A-induced proliferation of spleen cells through an increase in the glutathione level.[14] Ina *et al.* have shown that chronic low-dose-rate irradiation does not alter the total cell number of the spleen but augments the percentage of $CD4^+$ T cells and reduces the percentage of $CD40^+$ B cells in the spleens of mice.[7] In the present paper, we have shown that low-dose irradiation does not induce the proliferation of DCs, suggesting that low-dose irradiation has different effects depending on the kind of cells. Ina *et al.* have also reported that low-dose irradiation augments the expression of CD8 but does not alter the expression of CD 80 or CD86 in spleen cells.[7] These results are consistent with our results. In our experiments, the low-dose irradiation had no effect on the expression of the maturation markers of DCs, such as CD40, CD80, CD86, MHC class II, or CCR7, etc. These results suggest that the low-dose irradiation has no effect on the maturation of DCs. It is well known that helper T cells can be divided into at least 2 subsets (Th1 and Th2, etc.), and that Th1 is important for tumor immunity.[23–25] It has been reported that some DCs producing IL-12 induce Th1 from naïve helper T cells (DC1), while some DCs producing IL-10 induce Th2 from naïve helper T cells (DC2), and that Th1 is important for tumor immunity.[23,24] DCs derived from bone marrow hematopoietic stem cells and immature DCs differentiate into mature DCs (DC1 or DC2) under several kinds of stimulation, such as components of microbes, etc. These data suggest that the low-dose irradiation polarized the immature DCs in the spleen into DC1. These findings may explain the mechanisms underlying the effects of the low dose irradiation on tumor immunity.

In conclusion, low-dose irradiation activates T cells though the production of Th1 cytokines from DCs, resulting in the induction of Th1 cells from naïve T cells.

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