

「単球系細胞の分化誘導および機能に及ぼす放射線の影響」

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略語一覽

CD: cluster of differentiation

DAMPs: damaged-associated molecular patterns

DCs: dendritic cells

EDTA: ethylenediaminetetraacetic acid

FITC: fluorescein isothiocyanate

G-CSF: granulocyte colony-stimulating factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

HLA: human leukocyte antigen

HO-1: heme oxygenase-1

iDCs: immature dendritic cells

IFN: interferon

IL: interleukin

JNK: c-Jun N-terminal kinase

LPS: lipopolysaccharide

LPS-mDCs: DCs stimulated by LPS

mABs: monoclonal antibodies

MAP: mitogen-activated protein kinase

MCP-1: monocyte chemoattractant protein-1

mDCs: mature dendritic cells

MFI: mean fluorescence intensity

MHC: major histocompatibility complex molecules

MIP-1 β : macrophage inflammatory protein-1 β

MIX: a cytokine mixture (10-ng/mL rhTNF- α , 10-ng/mL rhIL-1 β , 10-ng/mL rhIL-6, and 1- μ g/mL PGE₂)

MIX-mDCs: DCs stimulated by MIX

MLR: mixed leukocytes reaction

MMP-9: matrix metalloproteinase-9

MyD88: myeloid differentiation factor 88

PAMPs: pathogen-associated molecular patterns

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PE: phycoerythrin

PMA: phorbol 12-myristate 13-acetate

PRRs: pattern recognition receptors

rh: recombinant human

ROS: reactive oxygen species

RT-PCR: reverse transcription polymerase chain reaction

TNF- α : tumor necrosis factor- α

Th: T helper

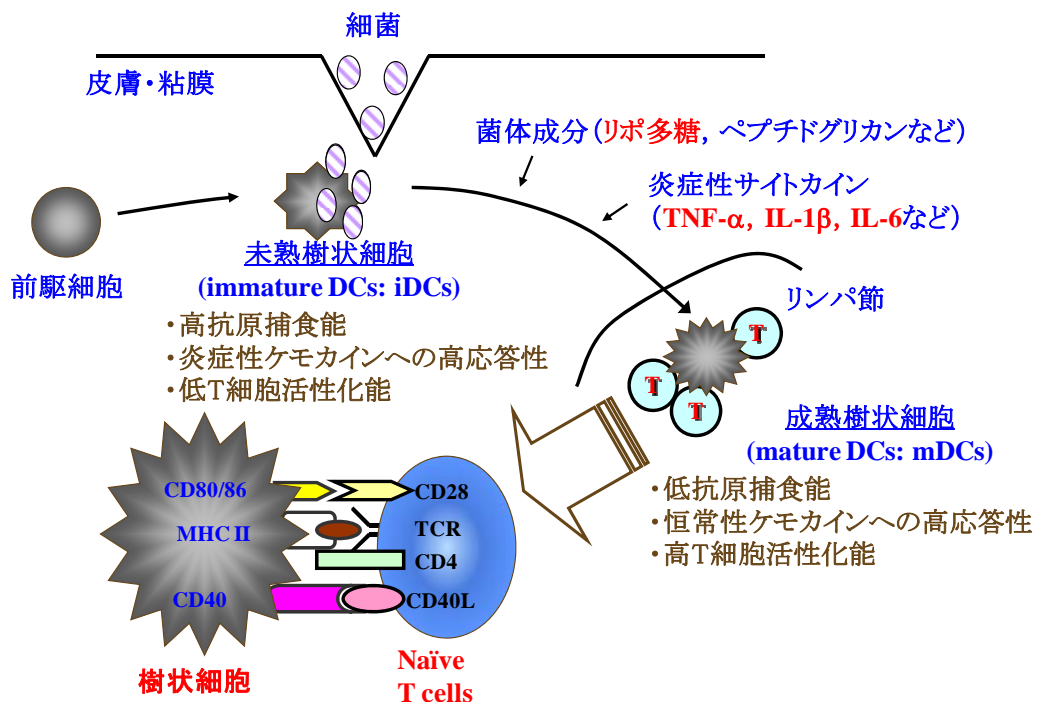
TLR: Toll-like receptor

TRIF: Toll/IL-1R domain-containing adaptor inducing IFN

序 論

単球は白血球の一種で、自然免疫を担当する。単球は末梢血中に存在するが、組織で樹状細胞やマクロファージに分化し、より強力な免疫応答能を獲得する。

樹状細胞は抗原提示細胞の一つで、免疫システムに中心的な役割を果たす。樹状細胞は骨髄細胞や単球などの前駆細胞から分化するが、未熟状態と成熟状態の2つの状態に大別される。樹状細胞の前駆細胞は皮膚や粘膜などの末梢組織に移動し、未熟樹状細胞として存在する。未熟樹状細胞は体内に侵入してきた病原体を感知し、貪食するとともに病原体由来の抗原をプロセッシングする。未熟樹状細胞は病原体成分や炎症性サイトカインの刺激により成熟化する。この成熟化に伴い高い抗原提示能を獲得し、それによりナイーブ T 細胞に効果的に病原体由来抗原を提示し獲得免疫を誘導する。従って、樹状細胞は自然免疫と獲得免疫を繋ぐ必要不可欠な免疫細胞である。



樹状細胞の高抗原提示能に着目し、樹状細胞を応用した抗腫瘍免疫誘導療法が期待されている。一方で、化学療法やがん放射線治療の治療後の再発予防などの予後を考える上で治療後の免疫力が非常に重要であるが、樹状細胞及びその前駆細胞に与える放射線の影響に関する報告は少ない。近年の研究により、分化した樹状

細胞は放射線による細胞死に対して抵抗性を示すこと、また放射線曝露樹状細胞では機能低下が起こることが明らかとなってきたが、樹状細胞の前駆細胞に及ぼす放射線の影響については以前不明のままである。

本研究では、樹状細胞の前駆細胞のヒト末梢血単球に着目し、ヒト末梢血単球由来樹状細胞の分化誘導における放射線の影響解明をすることで、がん放射線免疫療法の有効性向上を目指して行った。さらに、樹状細胞をはじめとする自然免疫担当細胞が病原体を認識する際に重要である病原体成分認識受容体の一種である Toll 様受容体の発現に及ぼす放射線の影響解明を行った。研究に先駆け、ヒト末梢血単球を用いた本研究は弘前大学大学院医学研究科倫理委員会より承認を得て実施した。本論文は以下に述べる一章から第三章で構成される。

《第一章》「X 線曝露ヒト単球から樹状細胞への分化誘導」

樹状細胞の前駆細胞であるヒト末梢血由来単球に X 線 5 Gy を照射し、サイトカイン存在下で未熟樹状細胞へ分化誘導を行ったところ、未熟樹状細胞に発現する細胞表面発現抗原の発現や未熟樹状細胞の特徴である貪食能が確認された。また、腫瘍壊死因子 α で刺激し、成熟樹状細胞へ誘導したところ、成熟樹状細胞のマーカーである CD83 などの発現が観察された。しかしながら、X 線照射によって未熟樹状細胞の培養上清中のマトリックスメタロプロテナーゼ-9 量が減少し、さらに成熟樹状細胞の T 細胞刺激能力が低下した。以上の結果より、放射線に曝露された単球からでも樹状細胞へ誘導できるものの、一部の機能低下が起きることが示された。

《第二章》「成熟刺激に依存した X 線曝露ヒト単球由来成熟樹状細胞の機能障害」

X 線曝露ヒト末梢血単球から誘導した未熟樹状細胞をグラム陰性細菌細胞壁構成成分のリポ多糖または炎症性サイトカインミックスで刺激し成熟樹状細胞へ誘導を行ったところ、リポ多糖刺激では、X 線照射群においてサイトカイン産生及び T 細胞刺激能力が著しく低下した一方で、炎症性サイトカインミックス刺激では X 線照射群での低下は認められなかった。以上の結果より、X 線曝露ヒト単球由来成熟樹状細胞の機能障害は成熟刺激に依存し、放射線によって外因性因子のリポ多糖に対する応答性は低

下するが、内因性因子の炎症性サイトカインミックスに対する応答性は比較的維持されていることが明らかとなった。

《第三章》「電離放射線は c-Jun N 末端キナーゼ活性を介してヒト単球系細胞株の Toll 様受容体 2 および 4 の発現を制御する」

病原体関連分子を認識する Toll 様受容体に及ぼす放射線の影響をヒト単球系細胞 THP1 を用いて検討したところ、未分化 THP1 細胞では X 線曝露後に Toll 様受容体 2 及び 4 の発現増強が起きるとともに、それぞれのアゴニスト誘導性の炎症性サイトカイン産生が増加した。一方で、THP1 由来マクロファージ様細胞では X 線照射によって Toll 様受容体 2 及び 4 の発現が低下したが、アゴニスト誘導性の炎症性サイトカイン産生は非照射細胞と同程度であった。以上の結果より、放射線がヒト単球系細胞の Toll 様受容体の発現に及ぼす影響は細胞分化に依存することが明らかとなった。さらに、THP1 細胞およびマクロファージ様細胞の恒常的な Toll 様受容体発現に c-Jun N 末端キナーゼ (JNK) が関与し、放射線が JNK の活性化制御を介して Toll 様受容体の発現を制御することが示唆された。

Chapter 1

Differential induction from X-irradiated human peripheral blood monocytes to dendritic cells

Abstract

Dendritic cells (DCs) are a type of antigen-presenting cell which plays an essential role in the immune system. To clarify the influences of ionizing radiation on the differentiation to DCs, we focused on human peripheral blood monocytes and investigated whether X-irradiated monocytes can differentiate into DCs. The non-irradiated monocytes and 5 Gy-irradiated monocytes were induced into immature DCs (iDCs) and mature DCs (mDCs) with appropriate cytokine stimulation, and the induced cells from each monocyte expressed each DC-expressing surface antigen such as CD40, CD86 and HLA-DR. However, the expression levels of CD40 and CD86 on the iDCs derived from the 5 Gy-irradiated monocytes were higher than those of iDCs derived from non-irradiated monocytes. Furthermore, the mDCs derived from 5 Gy-irradiated monocytes had significantly less ability to stimulate allogeneic T cells in comparison to the mDCs derived from non-irradiated monocytes. There were no significant differences in the phagocytotic activity of the iDCs and cytokines detected in the supernatants conditioned by the DCs from the non-irradiated and irradiated monocytes. These results suggest that human monocytes which are exposed to ionizing radiation can thus differentiate into DCs, but there is a tendency that X-irradiation leads to an impairment of the function of DCs.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells which stimulate naïve T cells and regulate immune responses.¹⁻³⁾ There are 3 stages of differentiation for DCs: DCs precursors, immature DCs (iDCs), and mature DCs (mDCs).¹⁻³⁾ iDCs locate in various tissues, where they capture antigens such as invading bacteria, viruses, or damaged tissue, and then process the antigens for presentation on major histocompatibility complex molecules (MHC). The pathogen-derived components or pro-inflammatory cytokines induce the DCs maturation. During the maturation process, the mDCs acquire a high antigen-presenting capacity instead of losing their phagocytotic activity, and vigorously stimulate T-cell responses.²⁾ This interaction results in immune activation or tolerance, depending on the phenotype and functional state of the involved DCs. In general, DCs are broadly classified into two types, plasmacytoid DCs and myeloid DCs. Plasmacytoid DCs are found primarily in blood and lymphoid organs and have the capacity to produce type I interferon (IFN).³⁾ On the other hand, myeloid DCs are found in many tissues.

Various applications of DCs to immunotherapy are now being investigated. The most striking application of DCs is the immunotherapy for cancer.⁴⁻⁷⁾ This immunotherapy utilizes the immunostimulatory ability of DCs for inducing antitumor immunity. On the other hand, DCs have also been applied to induce graft tolerance in hematopoietic stem cell transplantation or to induce tolerance in patients with autoimmune disease.³⁾ The tolerogenic or regulatory DCs, which cause immune tolerance, have been used to treat acute graft-versus-host disease and systemic inflammatory response.⁸⁻¹⁰⁾

For these applications, DCs are prepared from cluster of differentiation (CD) 34⁺ hematopoietic progenitors or CD14⁺ peripheral blood monocytes *ex vivo*.¹¹⁻¹⁶⁾ Especially, CD14⁺ peripheral blood monocytes are relatively easy to obtain in large quantities. However, it appears that the DCs derived from the monocytes of cancer patients are phenotypically and functionally inefficient in comparison to the DCs

derived from the monocytes of healthy donors.^{17, 18)} This is crucial problem when DCs are prepared from cancer patients. On the other hand, although radiotherapy and chemotherapy have been established as effective cancer therapies, whether the differential process for DCs is affected by these therapies remains to be elucidated.¹⁹⁾ Some previous reports have described that DCs derived from peripheral blood monocytes of healthy donors are resistant to ionizing radiation-induced cell death, but the DCs exposed to ionizing radiation are functionally weakened.²⁰⁻²³⁾ However, the influences of ionizing radiation on the DCs precursors have not yet been reported. This issue is important to consider the immune system of patients undergoing radiotherapy and the prognosis of patients who have undergone radiotherapy. In addition, the influences of ionizing radiation on the DCs precursors may also involve in the efficacy of immunotherapy using monocyte-derived DCs when monocytes are prepared from patients who have already undergone radiotherapy.

In this study, in order to clarify the influence of radiation on the differentiation in DCs, we focused on peripheral blood monocytes and investigated whether or not X-irradiated monocytes can differentiate into normal DCs.

2. Materials and methods

Reagents

The recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), interleukin-4 (rhIL-4) and tumor necrosis factor- α (rhTNF- α) were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). The fluorescence-labeled monoclonal antibodies (mABs), anti-human cluster of differentiation 1a-phycoerythrin (CD1a-PE), CD2-fluorescein isothiocyanate (CD2-FITC), CD40-FITC, human leukocyte antigen-ABC-FITC (HLA-ABC-FITC) and mouse IgG_{2a}-PE were purchased from Becton Dickinson (San Jose, CA, USA). Anti-human CD8-FITC, CD19-FITC, CD80-FITC, CD4-PE, CD14-PE, CD-11c-PE, CD83-PE, CD86-PE, mouse IgG₁-FITC, mouse IgG_{2b}-PE were purchased from Beckman Coulter (Fullerton, CA, USA), and

anti-human CD3-PE, HLA-DR-PE were purchased from eBioscience (San Diego, CA, USA) to use for the flow cytometer analysis. FITC-dextran (40,000 MW) was purchased from Molecular Probes, Inc. (Eugene, OR, USA) for the analysis of phagocytosis.

Collection of Monocytes from Buffy-Coat

This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from all of the normal human blood donors, peripheral blood was collected by the Aomori Red Cross Blood Center. The buffy-coat was prepared from whole-blood (400 ml) by the above-mentioned facility and was supplied to our research team. The peripheral blood mononuclear cells (PBMCs) were separated from the buffy-coat by centrifugation for 30 min at $400 \times g$ on a cushion of Lymphosepar I (1.077 g/ml; Immuno-Biological Laboratories Co. Ltd., Takasaki, Japan). After centrifugation, the PBMCs were washed three times with phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA). The monocytes were separated from the PBMCs according to the percoll density gradient-protocols published by Repnik *et al.*²⁴⁾ or adhering. When the PBMCs were more than 2.0×10^8 cells, the monocytes were separated from the PBMCs by centrifugation for 15 min at $580 \times g$ on a cushion of the hyper-osmotic percoll solution.²⁴⁾ After centrifugation, the monocyte fractions were collected, and washed with PBS containing 5 mM EDTA. Thereafter, the number of these cells was counted. In order to remove any obstacle platelets and dead cells, the monocytes fraction were centrifuged for 15 min at $350 \times g$ on a cushion of the iso-osmotic percoll solution.²⁴⁾ After centrifugation, the monocytes were collected and washed with PBS containing 5 mM EDTA. The purity of CD14⁺ monocytes was $64.6 \pm 7.6\%$ by a flow cytometer. On the other hand, when the PBMCs were less than 2.0×10^8 cells, the PBMCs were resuspended in PBS and allowed to adhere plastic dish (up to 1.0×10^7 cells per 60 mm dish) at 37°C for 1.5 – 2 h in a humidified atmosphere containing

5% CO₂. Thereafter, non-adherent cells were washed out twice with PBS.

The CD14⁺ monocytes or adherent monocytes were incubated in RPMI 1640 (GIBCO, Grand Island, NY, USA) supplemented with 2% heat-inactivated human AB serum (GEMINI BIO-PRODUCTS, Woodland, CA, USA) and 1% antibiotic-antimycotic (which hereafter we refer to as the medium). The concentration of CD14⁺ monocytes was up to 2.0×10^6 cells/ml.

In Vitro Irradiation of Monocytes

The radiation of X-rays (150 kVp, 20 mA, 0.5 mm Al and 0.3 mm Cu filters) was performed with an X-ray generator (MBR-1520R, Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm from the focus at a dose rate of 80.0-98.0 cGy/min.

In Vitro Generation and Culture of Human DCs

The DCs were generated from the monocytes according to previously published protocols¹⁴⁾ with some modification. In brief, non-irradiated and X-irradiated monocytes were cultured in the medium at 37°C overnight in a humidified atmosphere containing 5% CO₂. On the next day, 50 ng/ml rhGM-CSF and 50 ng/ml rhIL-4 were added to the medium and the monocytes were cultured to prepare the iDCs at 37°C for 5 days in a humidified atmosphere containing 5% CO₂. Because there was a possibility that some factors induced by X-irradiation may be removed from the culture medium by changing the culture medium with new medium, the culture medium was not changed with new medium during this 5-day culture. After 5 days of culture, the cells were harvested and an aliquot of the culture supernatants were collected and kept frozen at -85°C for the cytokine and zymography assay. Half of the culture medium was changed with new medium and the cells were re-cultured in the presence of 50 ng/ml rhTNF- α at 37°C for an additional 4 days in a humidified atmosphere containing 5% CO₂. After an additional 4 days of culture, the culture supernatants were also collected and kept frozen at -85°C for the cytokine and zymography assay.

Phenotypic Analysis

For the surface marker analysis of the iDCs and mDCs, the cells were stained with mABs conjugated to FITC or PE for 30 min at 4°C in the dark. The cells were also stained with corresponding FITC- or PE-conjugated isotype control mouse IgG. After 30 min, the cells were washed with cold PBS and analyzed by a flow cytometer (Epics XL, Beckman Coulter). The induced cells from monocytes could be distinguished from debris and lymphocytes using a region established by their high forward and side scatter signals. The cell surface phenotype of 1.0×10^4 cells within the region were analyzed. The data were expressed as the percent of positive cells or the mean fluorescence intensity (MFI).

Phagocytosis Assay

In order to determine the phagocytotic activity of the iDCs, FITC-dextran was added in a final concentration of 91 µg/ml to the cells (1.0×10^6 cells/ml), and the cells were incubated for 60 min at 37°C. As a negative control, the cells were put on ice in the presence of FITC-dextran. After incubation, the cells were washed twice with cold PBS and the FITC-dextran uptake of the iDCs was analyzed by a flow cytometer.

Allogeneic Mixed Leukocytes Reaction

Allogeneic 1.0×10^5 CD4⁺ T cells (> 98% CD4⁺ T cells), which were purified from PBMCs of three different individuals with the use of Human CD4 T lymphocyte Enrichment Set-DM (BD Bioscience), were co-cultured in 96-well flat bottom microplates (Asahi Techno Glass Co. Ltd., Chiba, Japan) with different numbers of mDCs. In order to prevent the proliferation of mDCs, the mDCs were 20 Gy-irradiated with an X-ray generator prior to co-culture with allogeneic CD4⁺ T cells. Both cells were co-cultured for 3 days in RPMI 1640 supplemented with 2% heat-inactivated human AB serum at 37°C in a humidified atmosphere containing 5% CO₂. After 3 days

of culture, the cells were incubated for additional 20 h in the presence of [^3H]-thymidine (1 $\mu\text{Ci}/\text{well}$, specific activity, 5 Ci/mmol; Moravek Biochemicals Inc, CA, USA). To determine CD4^+ T cells proliferation, the cells were harvested onto glass fiber filters (Whatman, England) with a semiautomatic harvester (Labo Mash, Labo Science, Tokyo, Japan), and then the amount of [^3H]-thymidine incorporation was measured by a liquid scintillation counter (LSC-5100, Aloka Co. Ltd., Tokyo, Japan). The CD4^+ T cells alone were cultured as a negative control. The experiment was performed in triplicate wells.

Cytokines Measurements

The cytokines in the culture supernatants released from the iDCs and mDCs were measured using the Bio-Plex protein array system (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 μl of sample.²⁵⁾ For the cytokine assays, we used premixed multiplex beads of the Bio-Plex human cytokine Th1/Th2 panel (Bio-Rad Laboratories), which included nine cytokines [IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, GM-CSF, IFN- γ , TNF- α]. Samples were analyzed in duplicate wells. The data were analyzed using the Bio-Plex Manager 4.0 software (Bio-Rad Laboratories). The detectable concentration of each cytokine was 2.0 pg/ml – 32 ng/ml.

Analysis of secreted MMP-9

The total matrix metalloproteinase-9 (MMP-9) in the culture supernatants of the iDCs and mDCs was analyzed by substrate zymography.²⁶⁾ The electrophoresis of each culture supernatant was carried out on a sodium dodecyl sulfate–polyacrylamide gel (10% acrylamide) containing 0.1% gelatin. Proteinases separated in the gels were renatured by gentle shaking in 50 mM Tris–HCl buffer (pH 7.5, containing 0.1 M NaCl and 2.5% Triton X-100) at room temperature for 1.5 h. The gels were then incubated in 50 mM Tris–HCl buffer (pH 7.5, containing 10 mM CaCl_2) at 37°C for 15 h, and

stained with Coomassie brilliant blue R-250. The bands were quantified by Image J analysis (1.37).

Statistical Analysis

The data are expressed as the mean values \pm SD. The comparisons of non-irradiation vs X-irradiation were performed by two-sided Mann-Whitney's *U*-test. The *p*-value was considered to be statistically significant if $p < 0.05$. The statistical analysis was performed using the Excel 2003 software program (Microsoft, USA) with the add-in software Statcel 2.²⁷⁾

3. Results

Phenotype of DCs

To clarify whether X-irradiated monocyte can differentiate into DCs, we attempted to induce iDCs from the human peripheral blood non-irradiated and 5 Gy-irradiated monocytes with a combination of rhGM-CSF plus rhIL-4. The cells derived from the non-irradiated monocytes had a typical dendritic morphology, which show a very large contact surface to their surroundings in comparison to the overall cell volume with the dendrites, under a microscope (data not shown). To analyze the cell surface phenotype of the iDCs, the induced cells were measured after gating on forward and side scatter by a flow cytometer (Fig. 1-[A]). The cells within this region showed no expression of any lineage-markers such as CD3 (T cells) and CD19 (B cells) (data not shown). Furthermore, these cells expressed high levels of CD11c, which is a myeloid-DCs marker (Fig. 1-[B]). These cells also expressed high levels of CD40, CD86, and HLA-DR, low levels of CD1a, and trace levels of CD14 (Fig. 1-[B]). Based on these results, the induced cells were thus determined to not be monocytes/macrophages, but DCs. Furthermore, these DCs also expressed low levels of CD80 and showed no expression of CD83 (Fig. 1-[B]), thus showing the characteristics of iDCs. The cells derived from the 5 Gy-irradiated monocytes also showed similar phenotype to the cells derived from the non-irradiated monocytes (Fig. 1-[C]).

Next, both types of iDCs were induced to mDCs in the culture with rhTNF- α . As shown in Fig. 2, both the cells induced from the non-irradiated and 5 Gy-irradiated monocytes expressed CD83, which is a DC's maturation marker.²⁸⁾ In addition, the expression of CD80, CD86 and HLA-DR on the stimulated cells were higher than those on the iDCs, showing the characteristics of mDCs. These results indicate that the induced cells from 5 Gy-irradiated monocytes are phenotypically iDCs and mDCs.

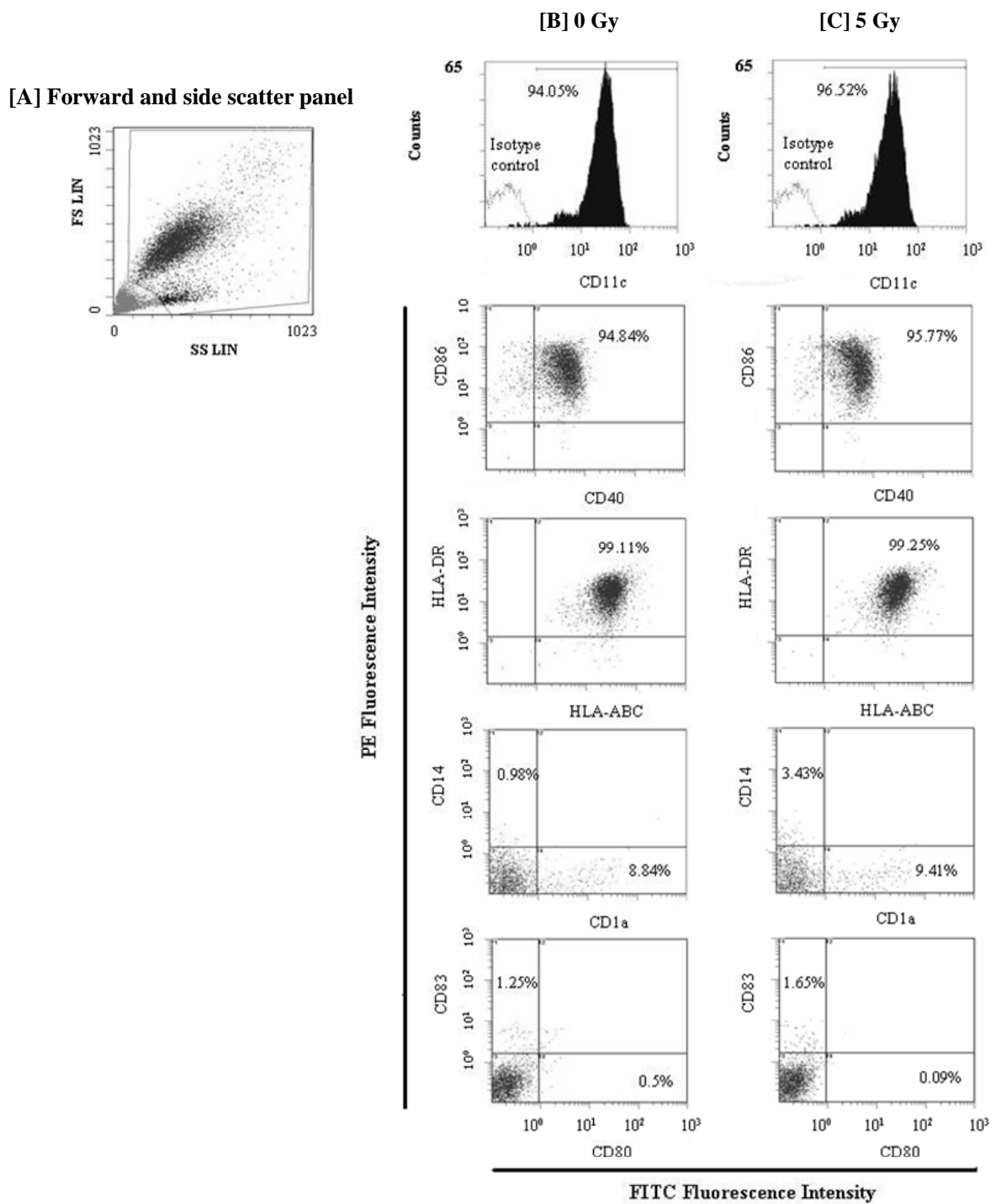


Fig. 1 Phenotypic characterization of iDCs by a flow cytometer. The non-irradiated and 5 Gy-irradiated monocytes were cultured with rhGM-CSF plus rhIL-4 for 5 days. After gating as indicated in the forward scatter (FS) and side scatter (SS) panel [A], the surface molecule expression of the cells derived from the non-irradiated [B] and 5 Gy-irradiated [C] monocytes were analyzed by a flow cytometer. The values in each quarter are the positive percentages of cell populations. The representative cytograms are shown.

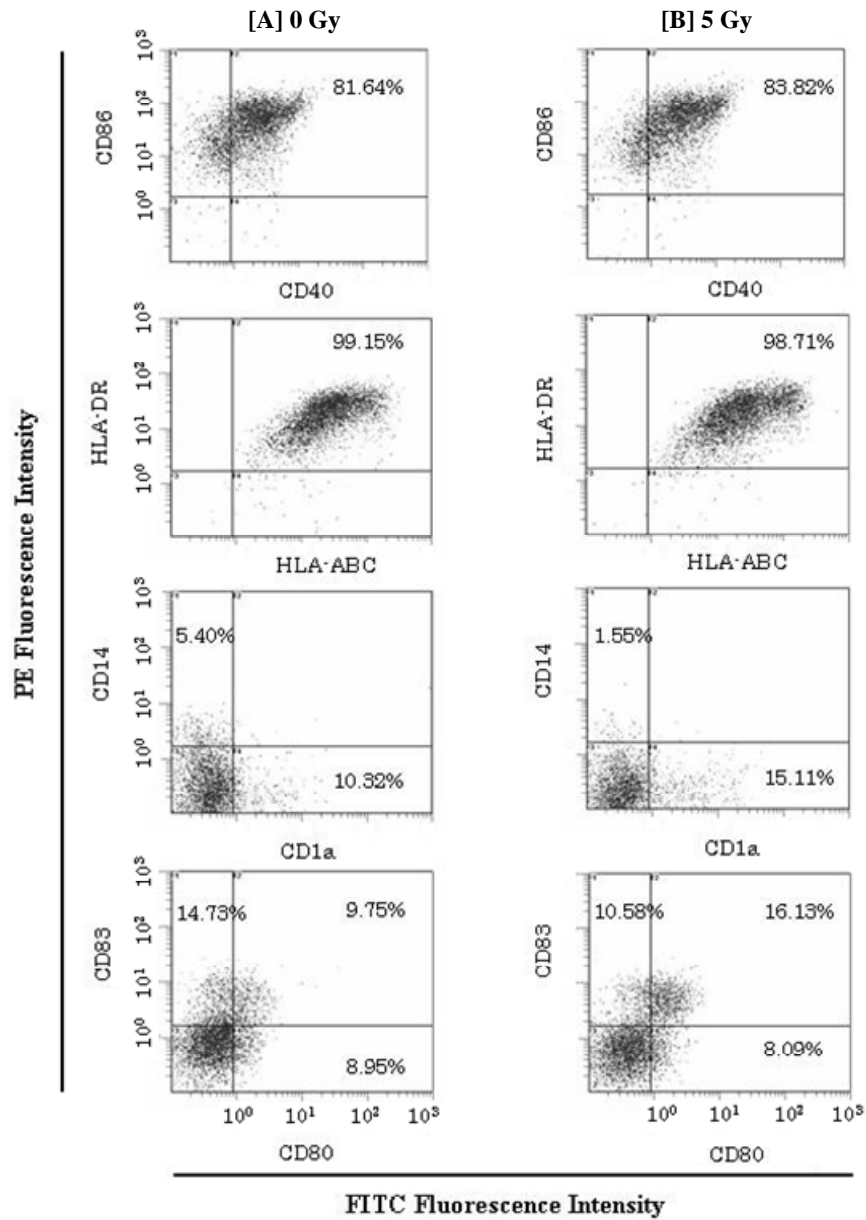


Fig. 2 Phenotypic characterization of mDCs by a flow cytometer. The cells, which were cultured with rhGM-CSF plus rhIL-4 for 5 days, were re-cultured in the presence of rhTNF- α for an additional 4 days. For determination of surface molecule expression, the cells derived from the non-irradiated [A] and 5 Gy-irradiated [B] monocytes were analyzed by a flow cytometer after forward and side scatter gating as indicated in Fig. 1-[A]. The values in each quarter are the positive percentages of cell populations. The representative cytograms are shown.

We investigated the influences of 5 Gy-irradiation to monocytes on the cell surface antigen expression in detail. As shown in Fig. 3-[A], the MFI of CD40 and CD86 on the iDCs derived from 5 Gy-irradiated monocytes was higher than that on the iDCs derived from non-irradiated monocytes. Although the MFI of CD40 on the mDCs derived from 5 Gy-irradiated monocytes was higher than that on the mDCs derived from non-irradiated monocytes, there was no significant difference in the CD86 expression on mDCs between non-irradiation and 5 Gy-irradiation (Fig. 3-[B]).

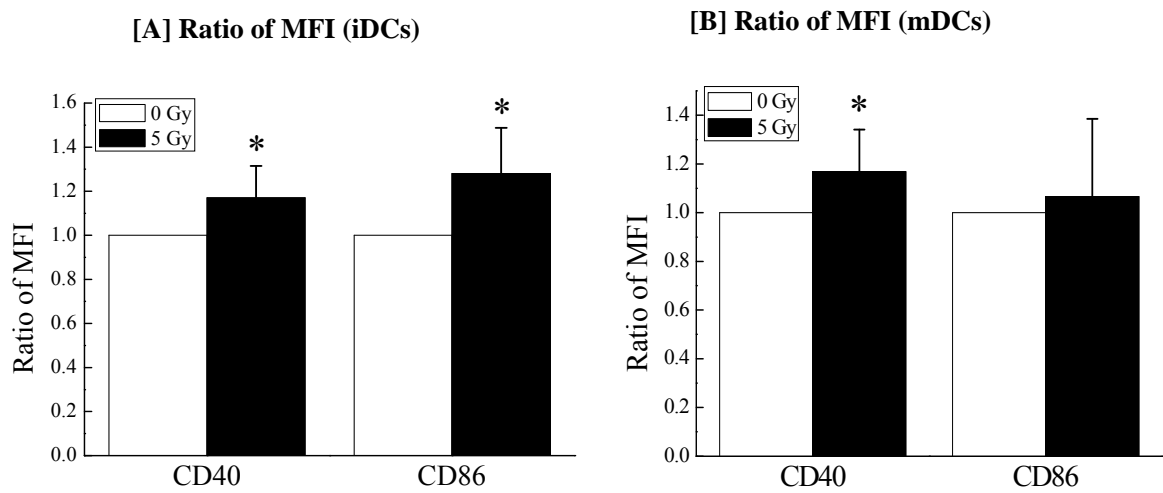


Fig. 3 Differences in the expression levels of the cell surface antigens between the DCs derived from non-irradiated and 5 Gy-irradiated monocytes. Ratios of CD40 and CD86 MFI, respectively, were obtained by calculating a ratio of the MFI of DCs derived from 5 Gy-irradiated monocytes to that of DCs derived from non-irradiated monocytes (5 Gy/ 0 Gy) in each individual. [A] The ratio of MFI on iDCs are expressed as the mean + SD of nine different individuals. [B] The ratio of MFI on mDCs are expressed as the mean + SD of 13 different individuals. * $p < 0.05$ by two-sided Mann-Whitney's U -test.

Function of DCs

The functional characteristics of the DCs were analyzed. As one of the characteristics of iDCs, it is known that iDCs have a high phagocytotic activity which thus allows them to capture antigens.^{1,2)} In the present study, the uptake of FITC-dextran was measured by a flow cytometer. The iDCs derived from non-irradiated monocytes showed an increase in

fluorescence intensity, thus demonstrating phagocytotic activity (Fig. 4-[A]). The iDCs derived from 5 Gy-irradiated monocytes showed less phagocytotic activity than the iDCs derived from non-irradiated monocytes in four out of six different individuals, and the large decrease (34% and 40%) was observed in two out of them (Fig. 4-[B]). However, there was no statistically significant difference in the phagocytotic activity between the non-irradiation and 5 Gy-irradiation. These results suggest that X-irradiation to monocytes relatively attenuate the ability of iDCs to capture antigens.

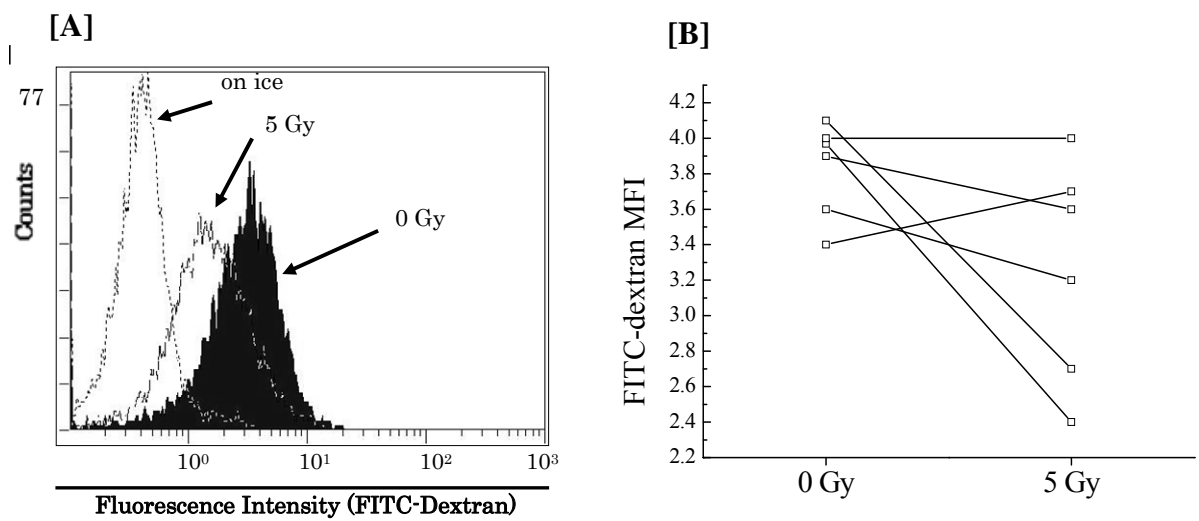


Fig. 4. The phagocytotic activity of iDCs. Phagocytosis was measured by the uptake of FITC-dextran. [A] A representative cytogram is shown. The uptake of FITC-dextran by the iDCs derived from the non-irradiated (filled gray histogram) and 5 Gy-irradiated (broken line histogram) monocytes was quantified by a flow cytometer. The dotted line histogram represents the negative control. [B] The data of six different individuals are shown as mean fluorescence intensity (MFI).

Since mDCs can stimulate the proliferation of allogeneic leukocytes and this ability is often used as a surrogate marker of their activation state, we next investigated the ability of the mDCs in allogeneic mixed leukocytes reaction (MLR) in seven different individuals. In order to quantify the CD4⁺ T cells proliferation, we measured the incorporation of [³H]-thymidine by CD4⁺ T cells. Both mDCs stimulated the proliferation of allogeneic CD4⁺ T cells (Fig. 5-[A]), although there was the large

difference in the incorporation of [^3H]-thymidine by CD4^+ T cells among individuals. Overall, the mDCs derived from 5 Gy-irradiated monocytes had about 32% less ability to stimulate allogeneic CD4^+ T cells compared with mDCs derived from non-irradiated monocytes (Fig. 5-[B]) when mDCs were co-cultured with CD4^+ T cells at a ratio of 1 : 10. These results suggest that X-irradiation to monocytes attenuate the interaction between mDCs and T cells.

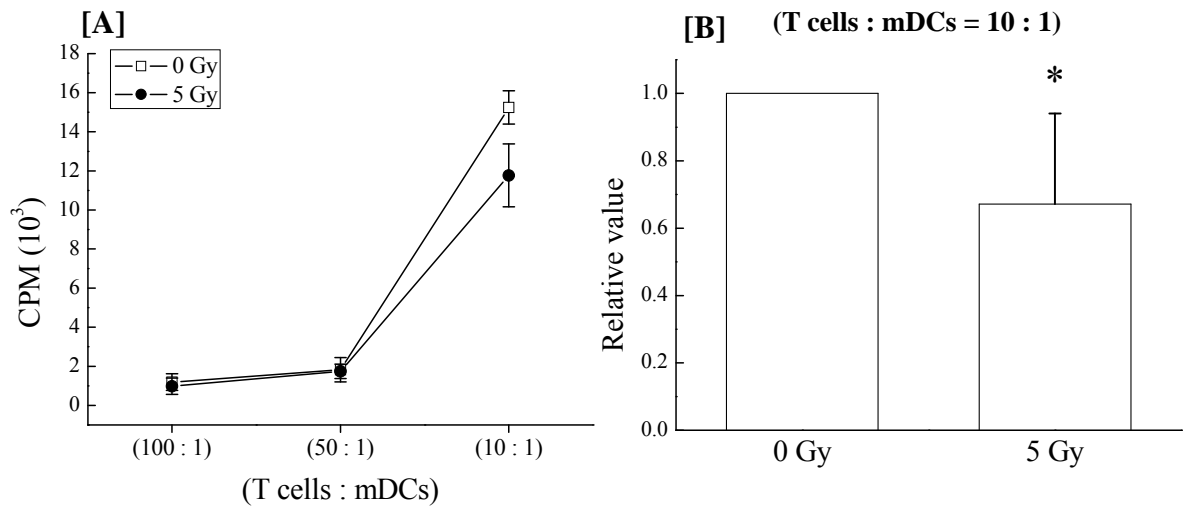


Fig. 5 The stimulating ability of mDCs on the allogeneic lymphocytes in a MLR. As we described in the materials and methods, each mDC was co-cultured with allogeneic CD4^+ T cells, and the proliferation of CD4^+ T cells was determined by the incorporation of [^3H]-thymidine. [A] The representative result of seven different individuals is shown. Values are the mean \pm SD obtained from triplicate cultures. The incorporation of [^3H]-thymidine by only CD4^+ T cells was less than 300 cpm. [B] A relative value of the proliferation (mean cpm obtained from triplicate cultures) was obtained by calculating the ratio of 5 Gy-irradiation to non-irradiation (5 Gy/ 0 Gy) in each individual. The data when mDCs were cultured with allogeneic CD4^+ T cells at a ratio of 1: 10 are shown and expressed as the mean + SD of seven different individuals. * $p < 0.05$ by two-sided Mann-Whitney's U -test.

Cytokines Production by DCs

mDCs produce various types of cytokines, which affect the T cell-mediated immune system. We thus next measured the cytokine production of the DCs in three different individuals. The cytokines released in the culture supernatants were assayed using

premixed multiplex beads, as described in the materials and methods. The culture supernatants of both iDCs contained large amounts of GM-CSF and IL-4 (data not shown). As shown in Fig. 6, IFN- γ , TNF- α and IL-10 were detected in the medium conditioned by both iDCs and mDCs derived from the non-irradiated monocytes. The medium conditioned by both iDCs and mDCs derived from the 5 Gy-irradiated monocytes also contained those cytokines (Fig. 6). The concentration of TNF- α in the culture supernatants of mDCs was more than 32 ng/ml (data not shown). Although IL-12 was not detected in the culture supernatants of the iDCs, it was detected in the culture supernatants of both mDCs (Fig. 6-[B]). However, no significant difference in the all cytokines concentrations tested in this study was observed between the non-irradiation and the 5 Gy-irradiation. These results indicate that DCs derived from 5 Gy-irradiated monocytes retain the capacity to produce cytokines tested here.

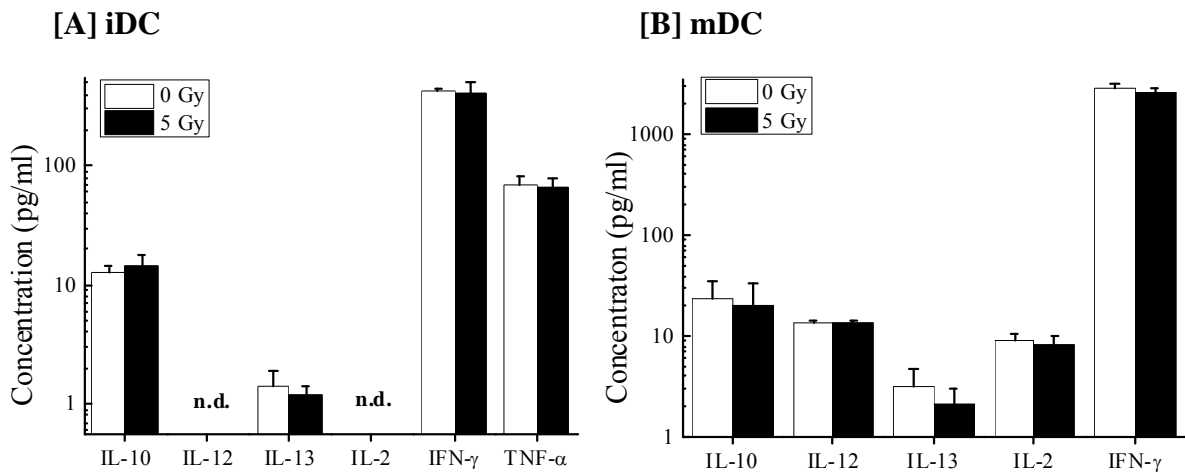


Fig. 6 Cytokines concentration detected in the culture supernatants of iDCs and mDCs. Each cytokine concentration in the culture supernatants of the iDCs [A] and mDCs [B] was measured using the Bio-Plex cytokine protein array system. The data are represented as the mean + SD of three different individuals. n.d. =not detectable (< 2 pg/ml).

Total MMP-9 in the Culture Supernatants of DCs

It is reported that DCs express several MMPs including MMP-9.^{29, 30)} MMP-9, which is gelatinase B, cleaves type IV collagen and contributes to DCs migration through the

extracellular matrix.^{29, 31)} We therefore investigated total MMP-9 in four different individuals. As shown in Fig. 7-[A], MMP-9 was presented in the culture supernatants of each DC. In agreement with previous reports,²⁹⁾ the total MMP-9 increased in the culture supernatants of the mDCs in comparison to the iDCs (Fig. 7-[B]). There was about 19% less total MMP-9 in the culture supernatants of iDCs derived from 5 Gy-irradiated monocytes compared with non-irradiation (Fig. 7-[B]). However, when the iDCs were induced to mDCs with rhTNF- α , this reduction disappeared.

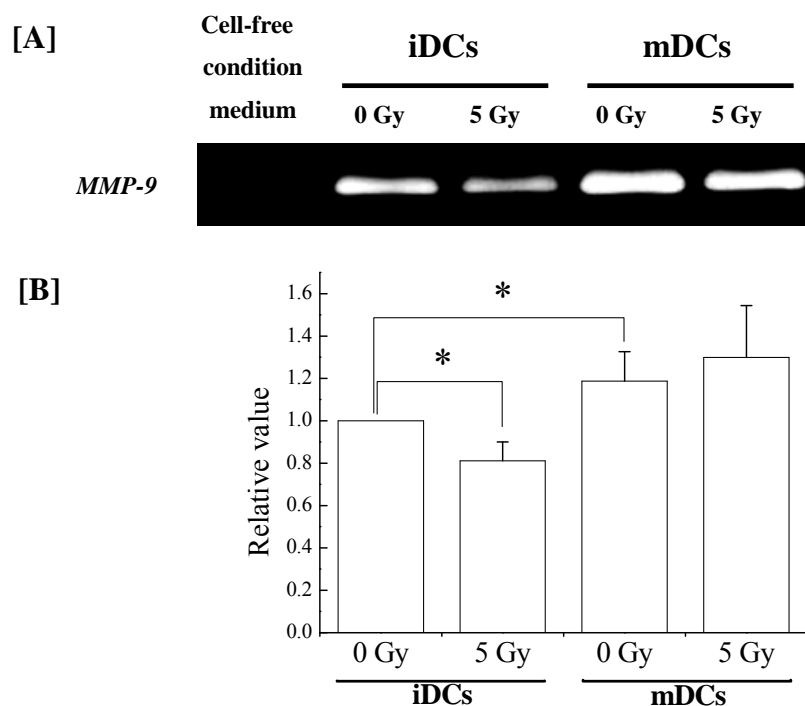


Fig.7 Total MMP-9 in the culture supernatants of iDCs and mDCs. The total MMP-9 in the culture supernatants of iDCs and mDCs derived from non-irradiated and 5 Gy-irradiated monocytes was analyzed by substrate zymography. [A] The data are representative of similar results obtained from four different individuals. [B] Quantification of bands was performed using Image J analysis. Each of data was divided by the value of iDCs derived from non-irradiation monocytes, respectively. The data are represented as the mean + SD of four different individuals. * $p < 0.05$ by two-sided Mann-Whitney's U -test.

4. Discussion

In this study, we evaluated the influences of X-irradiation on the differentiation from peripheral blood monocytes to DCs. We showed that X-irradiated monocytes can differentiate into iDCs and mDCs. We also showed that iDCs derived from 5 Gy-irradiated monocytes expressed higher levels of CD40 and CD86 compared with iDCs derived from non-irradiated monocytes (Fig. 3). Although few reports have described the influences of irradiation on the differential pathway from monocytes to DCs, previous studies have reported that ionizing radiation to iDCs caused a change in the expression of the surface antigens.²¹⁻²³⁾ Cao *et al.*²¹⁾ and Reuben *et al.*²²⁾ demonstrated that gamma irradiation reduced the expression of the CD86, CD80 and HLA-DR molecules on iDCs induced from human peripheral blood monocytes. On the other hand, Merrick *et al.* reported that the CD86 expression of iDCs was significantly up-regulated after gamma irradiation.²³⁾ Our results are consistent with Merrick's report regarding the up-regulation of co-stimulatory molecule expression, although there is a difference whether irradiation to monocytes or to iDCs. McBride *et al.* suggest the possibility that the “danger” signals induced by ionizing radiation may cause the maturation of DCs.¹⁹⁾ Their suggestion led us to consider that the various “danger” signals, which were induced either in the irradiated DCs-precursors or released from the irradiated bystander cells (including DCs-precursors and others), might influence the differentiation into DCs. TNF- α is an inflammatory cytokine and is one of the danger signals. Krivenko *et al.* reported that irradiation increased TNF production by human monocytes.³²⁾ One possibility is that X-irradiation performed on peripheral blood monocytes may induce TNF- α production, which directly influences the differentiation into DCs from monocytes. However, there is no significant difference between the tested cytokines including TNF- α in the supernatant conditioned by the non-irradiated and the 5 Gy-irradiated monocytes (Fig. 6-[A]), thus indicating no involvement of TNF- α . Further studies regarding other “danger” signals, for example reactive oxygen species produced by irradiated-cells¹⁹⁾ and the high-mobility-group box 1 protein

released from dying tumor cells³³⁾, are needed to clarify the mechanisms involved in the relationship between X-irradiation and the up-regulation of co-stimulatory molecule expression on iDCs. In this study, we used rhTNF- α as the maturation stimuli, and the mDCs derived from 5 Gy-irradiated monocytes expressed higher levels of CD40 compared with mDCs derived from non-irradiated monocytes (Fig. 3-[B]). However, this up-regulation of CD40 expression by 5 Gy-irradiation was not observed when we used lipopolysaccharide (LPS) as maturation stimuli in our progressing experiments (data not shown). These results indicate that the influences of X-irradiation to monocytes on the maturation of DCs depend on the types of maturation stimulus. It is thought that X-irradiation may influence the intracellular signal transduction induced by maturation stimuli, because the receptor of LPS is Toll-like receptor 4 and different from TNF receptor. Further studies regarding other maturation stimuli including LPS are now in progress.

In a functional analysis of the monocyte-derived DCs, no significant difference in the phagocytotic activity was observed between the non-irradiation and 5 Gy-irradiation, although this activity decreased by 5 Gy-irradiation in four out of six different individuals (Fig. 4-[B]). The result that the reduction of phagocytotic activity was not observed in all individuals may be owing to individual difference in the radiosensitivity. Regarding the stimulating ability of the mDCs on allogeneic T cell proliferation, although there was no difference in the MHC class II (HLA-DR) expression, which is involved in allogeneic MLR,^{28, 34)} the T cell proliferation by mDCs induced from the irradiated monocytes was reduced (Fig. 5). This result is consistent with previous studies that the stimulating capacity of DCs on T cell proliferation is reduced by applying ionizing radiation to the DCs,^{21, 23)} although there is a difference whether irradiation to monocytes or to DCs. Merrick *et al.* indicated that irradiated DCs were less effective in a MLR, and upon maturation produced significantly less IL-12 in comparison to the non-irradiated controls, while IL-10 secretion was maintained.²³⁾ On the other hand, Chauveau *et al.*³⁵⁾ demonstrated that human iDCs express heme

oxygenase-1 (HO-1), which is an intracellular antioxidant enzyme to degrade heme and inhibit immune responses,³⁶⁾ and that HO-1 expression is down-regulated by maturation stimuli. In addition, the induction of HO-1 not only inhibited the allostimulatory capacity of DCs but also suppressed IL-12 secretion of DCs, while it didn't affect IL-10 secretion of DCs.³⁵⁾ These two reports led to a hypothesis that oxidative stress such as ionizing radiation induce HO-1 in the monocytes and/or iDCs, and HO-1 suppress IL-12 secretion and allostimulatory ability of DCs. However, no detectable change in the IL-12 production by 5 Gy-irradiation was observed in our study (Fig. 6-[B]). Therefore, we think that other factors such as chemokine may be controlled by X rays-induced HO-1 in this case.³⁷⁾ In order to clarify these precise mechanisms, further experiments about the involvement of HO-1 are now in progress. On the other hand, zymographic analysis revealed that 5 Gy-irradiation induced a decrease of total MMP-9 during differentiation into iDCs from monocytes (Fig. 7). In contrast, 5 Gy-irradiation increased the ratio of mDCs to iDCs in the total MMP-9 activity. Lu *et al.* reported that addition of H₂O₂ to LPS-activated monocytes, but not naïve monocytes, caused a significant enhancement of MMP-1 production.³⁸⁾ We think that the reactive oxygen species generated by ionizing radiation enhance production of MMP-9 in the TNF- α -stimulated mDCs. However, it is unclear how X-irradiation induced a decrease of total MMP-9 during the differentiation into iDCs from monocytes.

In conclusion, the present data demonstrate that human peripheral blood monocytes exposed to ionizing radiation can differentiate into DCs, but some differences in their characteristics are observed between the DCs from non-irradiated monocytes and those from irradiated monocytes. Since the DCs derived from the monocytes of patients are phenotypically and functionally inefficient in comparison to healthy donors,^{17, 18)} the immune system in many cancer sufferers is thus considered to possibly be more severely damaged by radiotherapy or other therapies, such as chemotherapy during or after therapy. Furthermore, the efficacy of immunotherapy using monocyte-derived DCs against malignant tumors may be attenuated after

undergoing radiotherapy. Further understanding about the influences of ionizing radiation on immune cells including DCs will allow combination of radiotherapy and immunotherapy.³⁹⁾ At the start of radiotherapy or chemotherapy, the degree of damage to the DCs or DC precursors by various types of oxidative stress, including ionizing radiation, should therefore be monitored.

Chapter 2

Impairment of mature dendritic cells derived from X-irradiated human monocytes depends on the type of maturation stimulus used

Abstract

Dendritic cells (DCs) play an essential role in the immune system. We have showed that X-irradiated monocytes, precursors of DCs, can differentiate into DCs and then mature in terms of surface antigen expression after tumor necrosis factor- α stimulation while showing reduced functionality. DCs can mature in response to various types of maturation stimuli. Therefore, this study investigated whether DCs from monocytes exposed to ionizing radiation can adequately respond to pathogen-derived components and proinflammatory cytokines. Human monocytes separated from buffy coats were exposed to x-rays and were then differentiated into immature DCs (iDCs). iDCs were stimulated by lipopolysaccharide (LPS) or proinflammatory cytokine mixture (MIX). The DCs from nonirradiated and X-irradiated monocytes showed maturation after LPS and MIX stimulation as confirmed by findings of surface antigen expression. Upon LPS stimulation, however, the expression levels of CD80 and CD83 on the DCs from the X-irradiated monocytes were lower than those of the DCs from the nonirradiated monocytes. Such reductions were not observed upon MIX stimulation. Similarly, an impairment of matrix metalloproteinase-9 and cytokine production was observed in the LPS-stimulated DCs from the X-irradiated monocytes, whereas these impairments were not observed upon MIX stimulation. The ability of DCs to stimulate T cells was lower in the irradiated group compared with the nonirradiated group despite the type of maturation stimulus. Thus, the present results suggest that the influence of X-irradiation

on the maturation of DCs depends on the type of maturation stimulus used and that X-irradiation especially impairs the response of DCs to LPS.

1. Introduction

Dendritic cells (DCs) play key roles in immune regulation as professional antigen-presenting cells. They serve as a link between innate and adaptive immunity because they stimulate naive T cells more powerfully than other antigen-presenting cells. The development of DCs involves three stages of differentiation: DC precursors, immature DCs (iDCs), and mature DCs (mDCs).¹⁻³⁾ iDCs are present in various tissues, and they migrate to inflammatory sites. At inflammatory sites, iDCs capture antigens, such as invading bacteria and viruses, and then process the antigens for presentation on major histocompatibility complex molecules. Pathogen-derived components or proinflammatory cytokines can induce maturation of DCs. mDCs acquire a high antigen-presenting capacity and thereby vigorously stimulate T-cell responses. The powerful antigen-presenting capacity of DCs makes them useful for immunotherapy against cancer.^{40,41)}

The different classes of specific immune responses are driven by the biased development of pathogen-specific effector helper T cell subsets — that is, T helper 1 (Th1) and Th2 cells, that activate different components of cellular and humoral immunity. The polarization of Th-cell by DCs depends on the maturation stimuli that the DCs are exposed to.⁴²⁾ Recent reports propose that various types of stimuli including pathogen-derived components and proinflammatory cytokines can lead to the maturation of DCs.^{43,44)} As a representative pathogen-derived component, lipopolysaccharide (LPS) has been widely studied.⁴⁵⁻⁴⁷⁾ LPS is a major component of the outer membrane of Gram-negative bacteria and is recognized by the Toll-like receptor 4 (TLR4), which is a pattern recognition receptor.⁴⁸⁾ LPS strongly activates DCs, and activated DCs induce a Th1 immune response, which results in cell-mediated immunity including activation of cytotoxic T cells.⁴⁵⁻⁴⁷⁾ On the other hand, a combination of proinflammatory cytokines and prostaglandin E₂ (PGE₂), which mimics monocyte-conditioned medium, has been used as a maturation stimulus for processing DCs for cancer immunotherapy.⁴⁹⁾ However, it is controversial whether this

combination can induce the Th1 immune response because DCs stimulated by this combination produce low levels of interleukin-12 (IL-12).⁵⁰⁾ In general, it is considered that DCs stimulated by this combination induce a Th2 immune response from naive T cells, which results in a humoral immune response.⁵¹⁾

It is well known that T and B lymphocytes, which compose the adaptive immune system, are highly sensitive to ionizing radiation. On the other hand, DCs are relatively resistant to ionizing radiation-induced cell death, although DCs exposed to ionizing radiation are functionally weakened.²¹⁻²³⁾ We have previously showed that X-irradiated human monocytes, which are precursors of DCs, can differentiate into iDCs and then mature in terms of surface antigen expression after tumor necrosis factor- α (TNF- α) stimulation. However, the functions of mDCs derived from X-irradiated human monocytes, such as the stimulation of allogeneic T cells, are attenuated when compared with mDCs derived from nonirradiated monocytes.⁵²⁾ It remains unknown whether X-irradiation of human monocytes influences the maturation of DCs by stimuli such as pathogen-derived components and a proinflammatory cytokine mixture. This is an important issue to consider in an immune system that has been exposed to ionizing radiation. Furthermore, it is also important to determine the efficacy of immunotherapy using monocyte-derived DCs when monocytes are prepared from patients who have already undergone radiotherapy. Therefore, the present study investigated whether DCs derived from monocytes exposed to ionizing radiation can adequately respond to LPS and a proinflammatory cytokine mixture.

2. Materials and methods

Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), rhIL-1 β , rhIL-4, rhIL-6, and rhTNF- α were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). LPS (*Escherichia coli* 055:B5) and PGE₂ were purchased from Sigma Aldrich (St Louis, Mo, USA). The fluorescently labeled monoclonal antibodies (mABs)

anti-human cluster of differentiation 40-fluorescein isothiocyanate (CD40-FITC) and mouse IgG_{2a}-phycoerythrin (PE) were purchased from Becton Dickinson (San Jose, CA, USA). Anti-human CD19-FITC, CD80-FITC, CD11c-PE, CD14-PE, CD83-PE, CD86-PE, human leukocyte antigen-ABC-FITC (HLA-ABC-FITC), mouse IgG₁-FITC, and mouse IgG_{2b}-PE were purchased from Beckman Coulter (Fullerton, CA, USA), and the anti-human CD3-PE, HLA-DR-PE, and TLR4-PE for the flow cytometry were purchased from eBioscience (San Diego, CA, USA). The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Molecular Probes, Invitrogen Corporation (Eugene, Oregon, USA).

Collection of human monocytes from buffy coat

This study was approved by the committee of medical ethics of the Hirosaki University Graduate School of Medicine (Hirosaki, Japan). After obtaining informed consent from all of the healthy human blood donors, peripheral blood was collected by the Aomori Red Cross Blood Center. Peripheral blood mononuclear cells (PBMCs) were separated from the buffy coats by centrifugation with Lymphosepar I (1.077 g/mL; Immuno-Biological Laboratories Co. Ltd., Takasaki, Japan). The PBMCs were collected and washed at least three times with PBS(–) containing 5-mM EDTA. To obtain the monocyte fraction, the Human Monocyte Enrichment Set-DM (BD Bioscience, San Jose, CA, USA) was used for the negative selection of monocytes from PBMCs according to the manufacturer's instructions. The purity of CD14⁺ monocytes was > 70.0%, as determined by flow cytometry. To purify the monocyte fraction, the collected monocytes were resuspended in PBS(–) and allowed to adhere to a plastic dish at 37°C for 1.5 – 2 h in a humidified atmosphere containing 5% CO₂. Thereafter, nonadherent cells were removed by washing the dish twice with PBS(–).

The adherent monocytes were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 2% heat-inactivated human AB serum (Gemini Bio-products, Woodland, CA, USA) and 1% antibiotic-antimycotic (Gibco) (hereafter

referred to as the medium). The concentration of the monocytes was approximately 1.0×10^6 cells/mL.

In vitro irradiation of human monocytes

Irradiation with x-rays (150 kVp, 20 mA, 0.5-mm Al, and 0.3-mm Cu filters) was performed with an x-ray generator (MBR-1520R, Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm from the focus and at a dose rate of 98.0 – 104.0 cGy/min.

In vitro generation and culture of human DCs

DCs were generated from monocytes according to a previously published protocol with some modifications.⁵³⁾ Briefly, 20 h after X-irradiation of monocytes, 50 ng/mL rhGM-CSF and 50 ng/mL rhIL-4 were added to the medium, and the monocytes were cultured at 37°C for 5 days in a humidified atmosphere containing 5% CO₂ to prepare iDCs. On day 3 of this 5-day culture, half of the culture medium was changed with new medium containing rhGM-CSF and rhIL-4 (50 ng/mL). After 5 days of culture, the cells were harvested and counted by the trypan blue dye exclusion method. For the zymography assay, an aliquot of the culture supernatant was collected and kept frozen at –85°C. After 5 days of culture, half of the medium was changed with new medium and the cells were recultured in the presence of an LPS (1 µg/mL) or a cytokine mixture (10-ng/mL rhTNF-α, 10-ng/mL rhIL-1β, 10-ng/mL rhIL-6, and 1-µg/mL PGE₂: MIX) for 48 h.⁵⁴⁾ After 48 h, the cells were harvested and counted by the trypan blue dye exclusion method. Furthermore, the culture supernatants were also collected and kept frozen at –85°C for the zymography and cytokine assays. In some cases, the cells were stimulated for the indicated period.

Cell surface staining

For surface marker analysis of the iDCs and the mDCs, the cells were stained with mAbs conjugated to FITC or PE for 30 min at 4°C in the dark. The cells were also

stained with corresponding FITC- or PE-conjugated isotype control mouse IgGs. After 30 min, the cells were washed with cold PBS(–) and analyzed by a flow cytometer (Epics XL, Beckman Coulter). The cells induced from monocytes could be distinguished from debris and lymphocytes using a region established by their high forward and side scatter signals as shown in a previous report.⁵³⁾

Analysis of matrix metalloproteinase-9 activity

Total matrix metalloproteinase-9 (MMP-9) in the culture supernatants of iDCs and mDCs was analyzed by substrate zymography.⁵²⁾ Each culture supernatant was subjected to electrophoresis on a sodium dodecyl sulfate polyacrylamide gel (10% acrylamide) containing 0.1% gelatin. Proteinases separated in the gels were renatured by gentle shaking in 50-mM Tris-HCl buffer (pH 7.5, containing 0.1-M NaCl and 2.5% Triton X-100) at room temperature for 1.5 h. The gels were then incubated in 50-mM Tris-HCl buffer (pH 7.5, containing 10-mM CaCl₂) at 37°C for 15 h and stained with Coomassie Brilliant Blue R-250. The bands were quantified by an Image J analysis (1.37).

Measurement of intracellular reactive oxygen species production

The fluorescent probe H₂DCFDA was used for the measurement of intracellular reactive oxygen species (ROS). The cells were incubated for 15 min at 37°C, with 5-μM H₂DCFDA in PBS(–). After incubation, the cells were washed twice with PBS(–) and analyzed by flow cytometry.

Allogeneic mixed leukocyte reaction

Allogeneic CD4⁺ T cells numbering 1.0×10^5 cells (> 98% CD4⁺ T cells), which were purified from PBMCs with the Human CD4 T lymphocyte Enrichment Set-DM (BD Bioscience), were co-cultured in 96-well flat bottom microplates (Asahi Techno Glass Co. Ltd., Chiba, Japan) with 1.0×10^4 mDCs. The mDCs were 20 Gy-irradiated with an

X-ray generator before co-culture with allogeneic CD4⁺ T cells. Both cells were co-cultured for 4 days in RPMI 1640 medium supplemented with 2% heat-inactivated human AB serum. After 4 days of culture, the cells were incubated for an additional 20 h in the presence of [³H]-thymidine (1 μCi/well, specific activity, 5 Ci/mmol; Moravsek Biochemicals Inc, CA, USA). To determine CD4⁺ T cell proliferation, the cells were harvested onto glass fiber filters (Whatman, England) with a semiautomatic harvester (Labo Mash, Labo Science, Japan), and then the amount of [³H]-thymidine incorporation was measured by a liquid scintillation counter (LSC-5100, Aloka Co. Ltd., Japan).

Cytokine measurements

The cytokines in the culture supernatants were measured using the Bio-Plex protein array system (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well.⁵²⁾ For the cytokine assays, premixed multiplex beads of the Bio-Plex human cytokine 17-Plex panel (Bio-Rad Laboratories), which includes 17 cytokines [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), GM-CSF, interferon-γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), and TNF-α], were used. The samples were analyzed in duplicate. The data were analyzed using the Bio-Plex Manager 4.0 software (Bio-Rad Laboratories). The concentration of IL-5 was lower than the lowest detectable concentration (3.0 pg/mL).

Statistical analysis

The data are expressed as mean ± SD values. The comparisons between the nonirradiation and X-irradiation conditions were performed by two-sided Mann-Whitney's *U*-test or a two-sided paired *t*-test. A *p* < 0.05 was considered to be statistically significant. The statistical analysis was performed using the Excel 2003

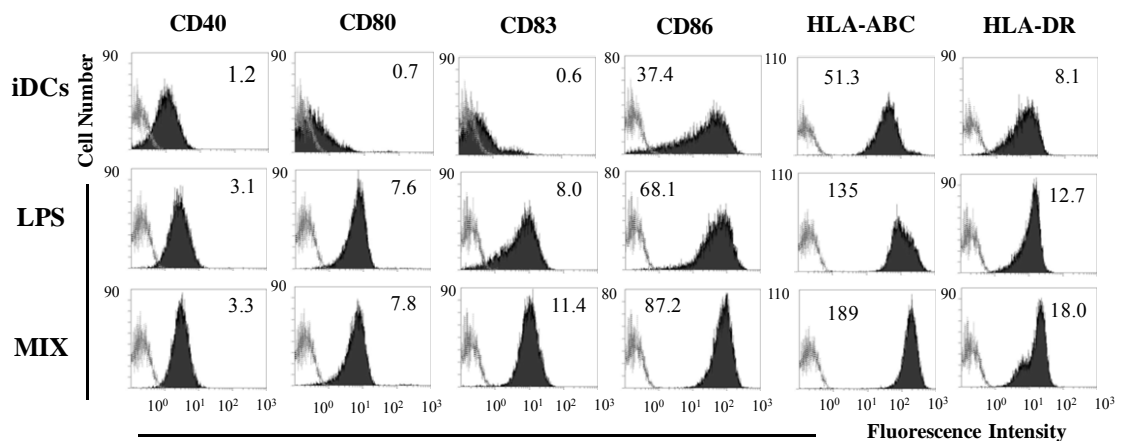
software program (Microsoft, USA) with the add-in software Statcel 2.²⁷⁾

3. Results

X-irradiation impairs the response of DCs to LPS in terms of cell surface antigen expression

To generate iDCs, nonirradiated or X-irradiated monocytes were cultured in the presence of rhGM-CSF plus rhIL-4. The iDCs derived from the nonirradiated monocytes expressed high levels of CD40, CD86, and HLA-DR (Fig. 8-[A]). However, they expressed no or trace levels of the co-stimulatory molecule CD80 and the DC maturation marker CD83,²⁸⁾ thus showing the characteristics of iDCs. When the iDCs from the nonirradiated monocytes were stimulated by LPS or MIX, the expressions of CD80 and CD83 were largely upregulated, suggesting that the cells had matured into mDCs (Fig. 8-[A]). Hereafter, we refer to DCs stimulated by LPS and MIX as LPS-mDCs and MIX-mDCs, respectively. Although no significant difference in number or viability of DCs was observed between the nonirradiation and X-irradiation conditions (Fig. 8-[B], [C]), the expressions of CD80 and CD83 in the LPS-mDCs from the X-irradiated monocytes were lower in a dose-dependent manner as compared with those of the LPS-mDCs from the nonirradiated monocytes (Fig. 8-[D]). On the other hand, no significant difference in the expression of these antigens between nonirradiation and X-irradiation was observed in the MIX-mDCs. These results show that X-irradiation impairs the response of DCs to LPS in terms of the expressions of maturation-related cell surface antigens.

Figure 8 [A]



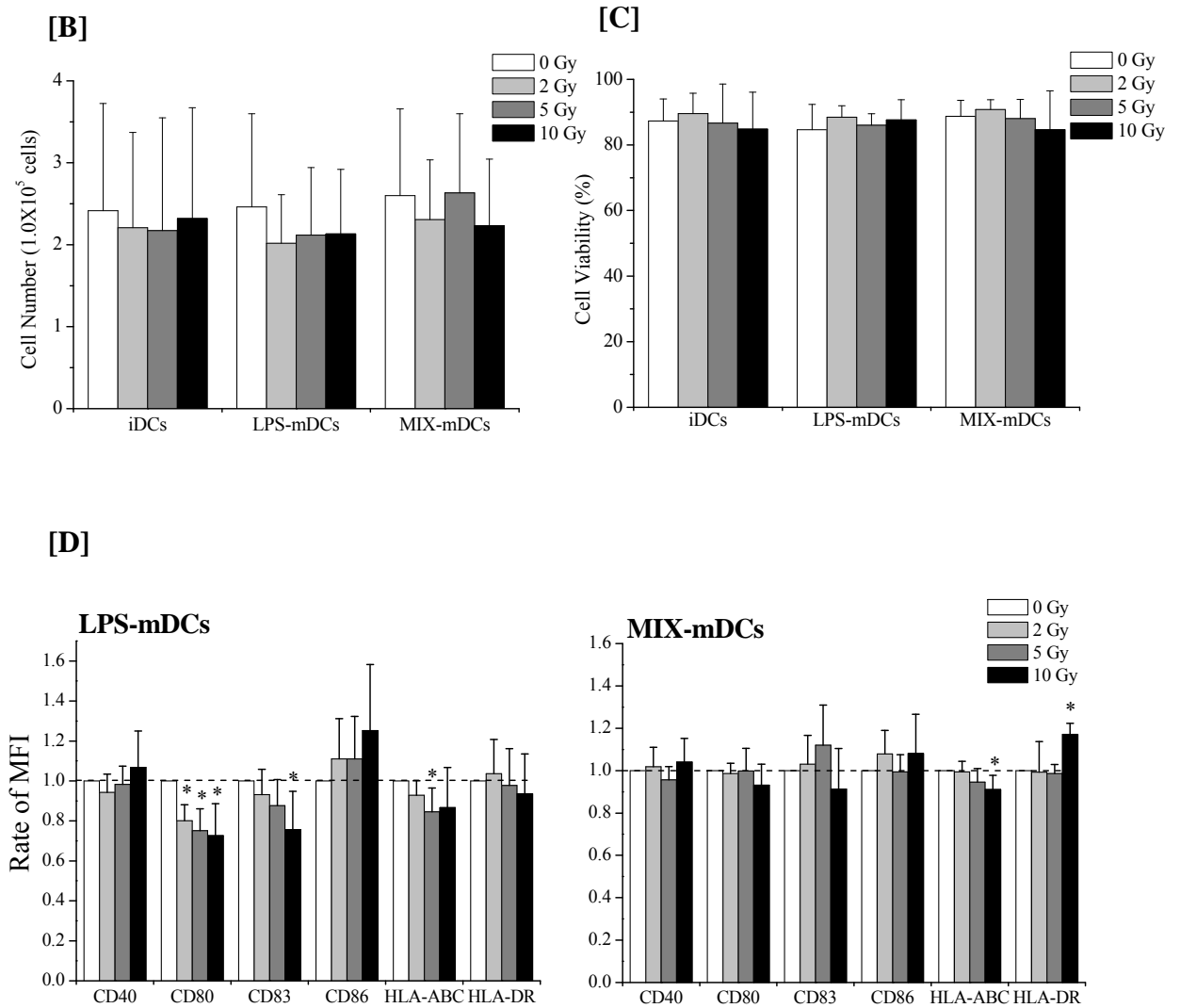


Fig. 8. Expression of cell surface antigens in the DCs stimulated by LPS and MIX. [A] To generate iDCs, rhGM-CSF and rhIL-4 were added to the medium 20 h after X-irradiation, and nonirradiated and X-irradiated monocytes were cultured for 5 days. The induced iDCs were stimulated by LPS or MIX for 48 h, and the phenotypic characteristics of the DCs were analyzed by flow cytometry. The representative histograms are shown. [B, C] Cell number and cell viability were estimated by the trypan blue dye exclusion method. The number of DCs obtained from 1.0×10^6 monocytes is shown. Cell viability was calculated as (the number of viable cells ÷ the number of viable cells and dead cells) $\times 100$. The data for the iDCs are from six different individuals. The data for LPS-mDCs and MIX-mDCs are from seven different individuals. [D] The mean fluorescence intensity (MFI) rate for each cell surface antigen is expressed as the mean \pm SD of more than four different individuals. LPS-mDCs or MIX-mDCs means DCs stimulated by LPS or MIX, respectively. * indicates $p < 0.05$ by two-sided Mann-Whitney's U -test.

X-irradiation impairs the response of DCs to LPS in terms of MMP-9 production

The activity of MMP-9 is important for the migration of DCs, as it degrades the extracellular matrix. The quantity of MMP-9 in the culture supernatants of the DCs was analyzed by substrate zymography. In line with our previous report,⁵²⁾ the MMP-9 activity of iDCs derived from the X-irradiated monocytes was lower than that of the iDCs derived from the nonirradiated monocytes (Fig. 9). Furthermore, MMP-9 activity was lower in the LPS-mDCs derived from the X-irradiated monocytes than in the LPS-mDCs derived from the nonirradiated monocytes. Although a reduction in MMP-9 activity was also observed in the mDCs derived by MIX stimulation of X-irradiated monocytes, the scale of reduction of MMP-9 activity was less than in mDCs derived by LPS stimulation of X-irradiated monocytes. These results show that X-irradiation largely impairs the response of DCs to LPS in terms of total MMP-9 production.

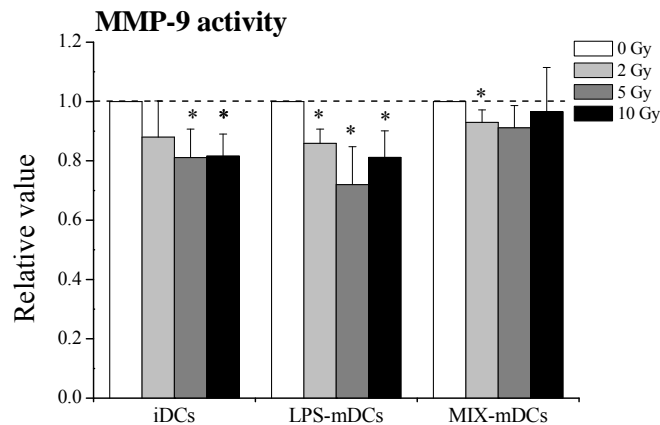


Fig. 9 Total MMP-9 activity of the iDCs or the DCs stimulated by LPS and MIX. To generate iDCs, rhGM-CSF and rhIL-4 were added to the medium 20 h after X-irradiation, and nonirradiated and X-irradiated monocytes were cultured for 5 days. The induced iDCs were stimulated by LPS or MIX for 48 h, and the culture supernatant of the DCs was harvested. The total amount of MMP-9 present in the culture supernatant of DCs was analyzed by substrate zymography. Quantification of bands was performed using Image J analysis. The relative value was obtained by calculating the ratio to the nonirradiated controls. The data represent the mean \pm SD of four different individuals. * indicates $p < 0.05$ by two-sided paired t -test.

X-irradiation impairs the allostimulatory ability of mDCs

mDCs can stimulate proliferation of allogeneic lymphocytes, and this activity is often used as a surrogate marker of their activation. LPS-mDCs and MIX-mDCs from nonirradiated monocytes had the ability to stimulate allogeneic T cell proliferation (Fig. 10). Irrespective of the type of maturation stimulus used, the allostimulatory ability of the mDCs derived from the X-irradiated monocytes was lower than those of the mDCs derived from the nonirradiated monocytes (Fig. 10). The downregulation of the allostimulatory ability of mDCs by X-irradiation was dose-dependent in LPS-mDCs but not in MIX-mDCs.

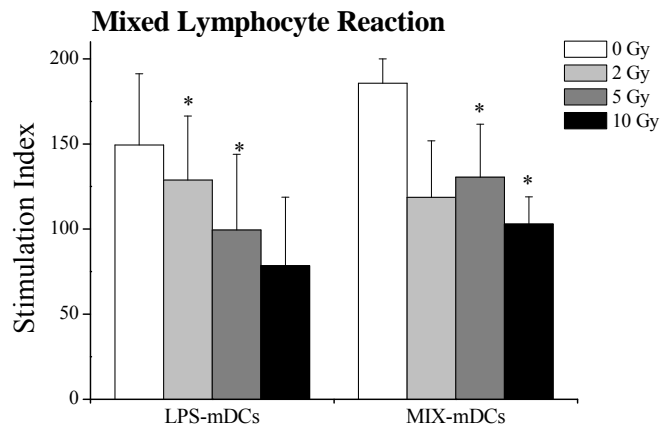


Fig. 10 Stimulatory ability of DCs on allogeneic T-cell proliferation. To generate iDCs, rhGM-CSF and rhIL-4 were added to the medium 20 h after X-irradiation, and nonirradiated and X-irradiated monocytes were cultured for 5 days. The iDCs were stimulated by LPS or MIX for 48 h. mDCs were 20 Gy-irradiated and were co-cultured with allogeneic T cells at a ratio of 1:10. T-cell proliferation was determined by the incorporation of [³H]-thymidine. The results are shown as a stimulation index, which is the ratio of count per minutes (cpm) in T cells in the presence of DCs to cpm of T cells in the absence of DCs. The data represent the mean \pm SD of three different individuals. * indicates $p < 0.05$ by two-sided paired t -test.

X-irradiation impairs the response of DCs to LPS in terms of cytokine production

The cytokine concentrations in the culture supernatants of the LPS-mDCs and MIX-mDCs are shown in Tables 1 and 2, respectively. The concentration of most cytokines (IL-2, IL-7, IL-8, IL-10, IL-12, IL-17, MCP1, G-CSF, IFN- γ , and MIP-1 β)

was higher in the culture supernatants of LPS-mDCs compared with those of MIX-mDCs. In LPS-mDCs, X-irradiation decreased the concentration of many cytokines, such as IL-10 and TNF- α . Furthermore, the concentration of IL-12 was significantly lower in the culture supernatants of the LPS-mDCs from the X-irradiated monocytes compared with that of the LPS-mDCs from the nonirradiated monocytes (Table 1). On the other hand, X-irradiation affected the production of only a few cytokines in MIX-mDCs. X-irradiation decreased the concentration of IL-13, whereas it increased the concentrations of IL-7 and MCP1 (Table 2).

X-irradiation does not affect the expression of TLR4 on iDCs

The TLR family is one of pattern recognition receptors, which recognizes pathogen-associated molecular patterns. To investigate the reasons for the reduced response of X-irradiated monocyte-derived iDCs to LPS, the expression of TLR4, a receptor for LPS, was analyzed by flow cytometry. The iDCs from the nonirradiated monocytes expressed cell surface TLR4. Furthermore, there was no difference in TLR4 expression between the iDCs derived from the nonirradiated and X-irradiated monocytes (Fig. 11-[A]).

X-irradiation changes the intracellular levels of ROS induced by maturation stimuli

Intracellular ROS are important for transduction of signals from TLR4 after LPS stimulation.⁵⁵⁾ Therefore, the intracellular ROS levels were analyzed. Irrespective of the type of maturation stimulus used, the ROS levels were lower in the DCs from the X-irradiated monocytes than in the DCs from the nonirradiated monocytes (Fig. 11-[B]). The ROS levels of the LPS-mDCs from the X-irradiated monocytes remained lower compared with those of the LPS-mDCs from the nonirradiated monocytes. However, the ROS levels of the MIX-mDCs from the X-irradiated monocytes partially recovered at later time points (24 and 48 h).

Table 1. Concentration of cytokines in the culture supernatants of LPS-mDCs

		0 Gy	2 Gy	5 Gy	10 Gy
		Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
IL-1 β	pg/mL	96.7 \pm 30.5	107 \pm 42.5	114 \pm 52.5	109 \pm 39.0
	Ratio		1.09 \pm 0.13	1.16 \pm 0.23	1.13 \pm 0.14
IL-2	pg/mL	40.8 \pm 0.9	35.8 \pm 3.5	33.3 \pm 5.80	36.4 \pm 3.4
	Ratio		0.88 \pm 0.07 *	0.81 \pm 0.13 *	0.89 \pm 0.07 *
IL-6	pg/mL		>31270		
IL-7	pg/mL	14.3 \pm 2.6	13.1 \pm 4.0	14.3 \pm 3.6	14.5 \pm 3.6
	Ratio		0.92 \pm 0.18	1.00 \pm 0.16	1.01 \pm 0.13
IL-8	pg/mL	10220 \pm 1674	1043 \pm 3282	8353 \pm 1907	10786 \pm 3019
	Ratio		1.00 \pm 0.16	0.82 \pm 0.13 *	1.06 \pm 0.31
IL-10	pg/mL	568 \pm 371	381 \pm 274	404 \pm 333	424 \pm 330
	Ratio		0.65 \pm 0.08 *	0.65 \pm 0.20 *	0.74 \pm 0.24 *
IL-12	pg/mL	255 \pm 129	87.5 \pm 42	60.3 \pm 24.6	75.7 \pm 44.8
	Ratio		0.36 \pm 0.06 *	0.26 \pm 0.06 *	0.31 \pm 0.08 *
IL-13	pg/mL	7.5 \pm 5.2	4.2 \pm 2.3	3.6 \pm 2.2	2.9 \pm 1.5
	Ratio		0.67 \pm 0.28	0.54 \pm 0.16 *	0.46 \pm 0.15
IL-17	pg/mL	105 \pm 2.7	92.7 \pm 6.7	88.0 \pm 13.2	96.7 \pm 6.8
	Ratio		0.88 \pm 0.08	0.84 \pm 0.14 *	0.92 \pm 0.08 *
MCP1	pg/mL	10764 \pm 6725	8839 \pm 4837	5783 \pm 1100	8990 \pm 2644
	Ratio		0.87 \pm 0.31	0.64 \pm 0.23	1.00 \pm 0.38
G-CSF	pg/mL	635 \pm 488	466 \pm 426	443 \pm 411	454 \pm 409
	Ratio		0.69 \pm 0.11 *	0.64 \pm 0.15 *	0.68 \pm 0.13 *
IFN- γ	pg/mL	1345 \pm 19.7	1146 \pm 65.9	1091 \pm 190	1173 \pm 102
	Ratio		0.85 \pm 0.06 *	0.81 \pm 0.14 *	0.87 \pm 0.08 *
TNF- α	pg/mL	32612 \pm 3379	25149 \pm 5978	22425 \pm 7206	25058 \pm 5183
	Ratio		0.76 \pm 0.13 *	0.68 \pm 0.19 *	0.76 \pm 0.11 *
MIP-1 β	pg/mL		>24075		

The unit measure of cytokine concentration is pg/mL. As rhGM-CSF and IL-4 were added to the culture medium, the concentrations of these cytokines are not shown. When the cytokine concentrations contained in each culture supernatant exceeded the detectable upper limits, the lowest detected concentration is shown (in brief, > the lowest detected concentration). The concentration ratio was obtained by dividing the concentration of each cytokine in the X-irradiated cells to the concentration in the nonirradiated controls. The data of four different individuals are shown. * indicates $p < 0.05$ by two-sided Mann-Whitney's U -test.

Table 2. Concentration of cytokines in the culture supernatants of MIX-mDCs

		0 Gy	2 Gy	5 Gy	10 Gy
		Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
IL-2	pg/mL	21.4 \pm 1.8	22.4 \pm 2.8	21.3 \pm 2.9	22.2 \pm 2.4
	Ratio		1.04 \pm 0.09	0.99 \pm 0.05	1.03 \pm 0.03
IL-7	pg/mL	6.2 \pm 1.2	7.5 \pm 2.6	8.1 \pm 3.0	8.2 \pm 2.8
	Ratio		1.19 \pm 0.20	1.28 \pm 0.23 *	1.30 \pm 0.25 *
IL-8	pg/mL	7004 \pm 2984	7214 \pm 3526	6685 \pm 2400	6241 \pm 1489
	Ratio		1.01 \pm 0.17	0.97 \pm 0.12	0.95 \pm 0.24
IL-10	pg/mL	5.7 \pm 0.4	4.8 \pm 0.6	5.0 \pm 0.4	5.5 \pm 1.2
	Ratio		0.85 \pm 0.10 *	0.88 \pm 0.13	0.98 \pm 0.24
IL-12	pg/mL	14.1 \pm 8.9	15.4 \pm 11.8	12.7 \pm 8.6	13.5 \pm 9.2
	Ratio		1.04 \pm 0.12	0.90 \pm 0.10	0.96 \pm 0.12
IL-13	pg/mL	6.3 \pm 3.4	3.3 \pm 1.1	2.8 \pm 1.1	2.2 \pm 0.6
	Ratio		0.58 \pm 0.17 *	0.46 \pm 0.07 *	0.41 \pm 0.15 *
IL-17	pg/mL	55.8 \pm 3.5	58.5 \pm 7.2	57.6 \pm 3.4	57.9 \pm 6.5
	Ratio		1.05 \pm 0.09	1.03 \pm 0.03	1.04 \pm 0.08
MCP1	pg/mL	1858 \pm 1545	2619 \pm 2351	2731 \pm 2414	3547 \pm 2827
	Ratio		1.37 \pm 0.12 *	1.50 \pm 0.23 *	2.02 \pm 0.32 *
G-CSF	pg/mL	49.1 \pm 21.3	47.0 \pm 19.2	44.6 \pm 15.6	45.7 \pm 19.3
	Ratio		0.96 \pm 0.09	0.93 \pm 0.13	0.93 \pm 0.09
IFN- γ	pg/mL	722 \pm 62	732 \pm 88	726 \pm 47	735 \pm 78
	Ratio		1.01 \pm 0.08	1.01 \pm 0.03	1.02 \pm 0.03
MIP-1 β	pg/mL	688 \pm 262	729 \pm 183	769 \pm 174	756 \pm 82
	Ratio		1.09 \pm 0.16	1.16 \pm 0.15	1.18 \pm 0.29

The unit of cytokine concentration is pg/mL. As rhGM-CSF, IL-4, IL-1 β , IL-6, and TNF- α were added to the culture medium, the concentrations of these cytokines are not shown. The concentration ratio was obtained by dividing the concentration of each cytokine in the X-irradiated cells to the concentration in the nonirradiated controls. The data of four different individuals are shown. * indicates $p < 0.05$ by two-sided Mann-Whitney's U -test.

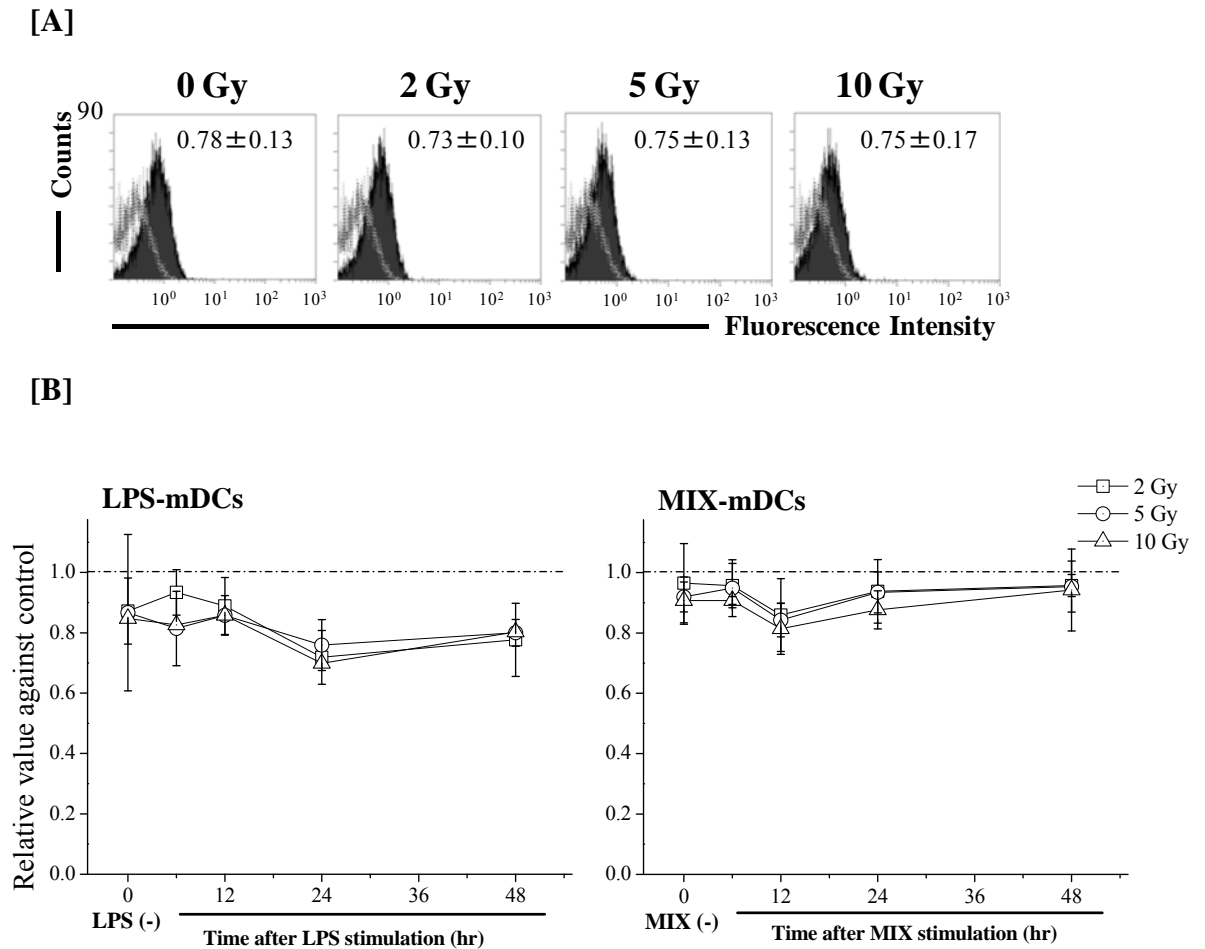


Fig. 11. TLR4 expression of iDCs and kinetics of intracellular ROS levels of DCs after stimulation by LPS or MIX. [A] Cell surface TLR4 expression was analyzed by flow cytometry. Representative histograms are shown. The dotted line indicates the isotype control. The MFI is shown in the inset. The data are presented as mean \pm SD of four different individuals. [B] Kinetics of intracellular ROS levels after stimulation by LPS and MIX. The intracellular ROS levels were analyzed using H₂-DCFDA. The intracellular ROS levels of the iDCs or the LPS-mDCs and MIX-mDCs for the indicated period were analyzed by flow cytometry. The relative value was obtained by calculating the ratio of MFI for H₂-DCFDA to the nonirradiated controls at each time point. The data are expressed as the mean \pm SD of from two to three different individuals.

4. Discussion

In this study, we demonstrated that DCs derived from X-irradiated monocytes can develop into mDCs in response to LPS or MIX stimulation. However, the expressions of maturation-related cell surface antigens, the MMP-9 production, the inflammatory cytokine production, and the functionality of the LPS-mDCs derived from the X-irradiated monocytes were lower than those of the LPS-mDCs derived from the nonirradiated monocytes. However, the response of the DCs derived from the X-irradiated monocytes to the MIX stimulation was largely similar to that of the DCs from the nonirradiated monocytes.

Previous studies demonstrated that iDCs exposed to high doses of ionizing radiation show a low response to LPS.²³⁾ However, exposure to low doses of ionizing radiation (< 2 Gy) during the differentiation process has been shown to have no effect on the behavior of DCs stimulated by LPS.⁴⁷⁾ Furthermore, Reuben et al. reported that DCs exposed to ionizing radiation during differentiation into iDCs showed no defect in phenotypic or functional characteristics after MIX stimulation.²²⁾ In line with these data, the DCs from the X-irradiated monocytes showed a low response to the LPS but not to the MIX stimulation. However, the impairment of the DCs derived from the X-irradiated monocytes to stimulate T-cell proliferation was observed upon LPS and MIX stimulation (Fig. 10). In the report by Reuben et al.,²³⁾ ionizing radiation was delivered in the presence of cytokines required for the differentiation of DCs, whereas in the present study, radiation was delivered under exogenous cytokine-free conditions. As cytokines have multiple effects including anti-apoptotic effects,^{56,57)} it is likely that the presence of cytokines during irradiation may have attenuated the damages of irradiation. Although it remains unknown why DCs derived from X-irradiated monocytes largely retain their response to MIX stimulation, we speculate that the effects of irradiation are compensated for by a combination of multiple factors because DCs derived from X-irradiated monocytes show some functional impairment after stimulation by TNF- α alone.⁵²⁾

In the present study, the DCs from the X-irradiated monocytes showed a reduced response to LPS in terms of the expression of maturation-related antigens, functional characteristics, and intracellular ROS levels (Figs. 8-[D] to 11-[B] and Table 1). The TLR4 expression of iDCs was analyzed because it is essential for LPS recognition.⁴⁸⁾ However, no significant difference was observed between the nonirradiated and X-irradiated cells (Fig. 11-[A]), thus indicating that the low response of DCs from X-irradiated monocytes is not due to a difference in TLR4 expression. It has been reported that LPS-induced ROS generation and cellular redox status are involved in the maturation of DCs.⁵⁸⁾ Furthermore, in TLR4-mediated signal transduction, ROS are important mediators of LPS-induced p38 activation.⁵⁵⁾ Nakahara et al. reported that a p38 mitogen-activated protein kinase inhibitor profoundly inhibited phenotypic maturation, cytokine production (such as IL-12 p70 and TNF- α), and the allostimulatory ability of DCs stimulated by LPS.²⁸⁾ As the biological effects of X-irradiation are largely indirectly mediated through ROS, it is thought that ROS generated by ionizing radiation changes the cellular redox status, which may in turn result in the low response to LPS through modification of ROS-mediated signal transduction. Chauveau et al. demonstrated that iDCs express the antioxidant enzyme heme oxygenase-1 (HO-1) and that LPS stimulation downregulates HO-1 expression and increases ROS levels.³⁵⁾ Although the iDCs expressed HO-1 at the protein level in our preliminary experiments, no difference was observed in the HO-1 expression levels between the nonirradiated and X-irradiated conditions (data not shown). Therefore, another redox system, such as the glutathione or thioredoxin redox system, might have been involved in the production of low levels of ROS in the DCs derived from the X-irradiated monocytes. Because the secretory molecule MD-2 plays a principal role in LPS recognition,⁶⁰⁾ the possibility that the TLR4-MD-2 complex is affected by ionizing radiation cannot be ruled out.

In conclusion, the present study indicates that the influence of X-irradiation of monocytes on the maturation of DCs depends on the type of maturation stimulus used.

Furthermore, the DCs derived from the X-irradiated monocytes showed a lower response to LPS as compared with the DCs derived from the nonirradiated monocytes. Recent immunotherapeutic research has been focused on the use of DCs as potential cellular vaccines against malignant tumors.^{40,41)} Therefore, although it is necessary to resolve the problem of inducing Th1 cells using MIX-stimulated DCs, MIX stimulation may be preferable in immunotherapy using monocyte-derived DCs after radiotherapy.

Chapter 3

Ionizing radiation affects the expression of Toll-like receptors 2 and 4 in human monocytic cells through c-Jun N-terminal kinase activation

Abstract

Pattern recognition receptors recognize pathogen-associated molecular patterns. Among these, Toll-like receptors (TLRs) have well-characterized roles in antibacterial and antiviral immunity. In the present study, the effects of ionizing radiation on the expression of TLRs and cellular responses to ligands were investigated in THP1 monocytes (human monocytic leukemia cells) and THP1-derived macrophage cells (macrophage-like cells), which are induced by culturing in the presence of phorbol 12-myristate 13-acetate. TLR2 and TLR4 expression was detected in THP1 and macrophage-like cells, X-irradiation caused increased expression of these TLRs in THP1 and decreased expression in macrophage-like cells. Responses to FSL-1 (TLR2 ligand) and lipopolysaccharide (LPS, TLR4 ligand) were estimated by determining the induction of tumor necrosis factor- α (TNF- α). After FSL-1 or LPS stimulation, TNF- α induction was greater in X-irradiated THP1 monocytes than in non-irradiated cells. However, although TNF- α expression was not affected by X-irradiation in macrophage-like cells, the expression of LPS-inducible interferon- β was lower following X-irradiation of macrophage-like cells. To clarify the mechanisms of TLR2 and TLR4 regulation by X-irradiation, expression of mitogen-activated protein kinase was investigated. These experiments showed that c-Jun N-terminal kinase (JNK) mediated increases in TLR expression in X-irradiated THP1 monocytes, and decreases in TLR expression in X-irradiated macrophage-like cells. This study demonstrates that

ionizing radiation modulates ligand-responsive TLR expression through the JNK pathway depending on differentiation state.

1. Introduction

Adaptive immunity involves antigen-specific immune responses of T and B cells, whereas innate immunity is mediated by antigen-presenting cells such as macrophages and dendritic cells (DCs), which initiate adaptive immune responses following pathogen recognition.^{61,62)} The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). To date, several PRRs have been identified, including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors, and nucleotide-binding oligomerization domain-like receptors.⁶³⁻⁶⁶⁾

TLRs are well-studied PRRs that are indispensable for antibacterial and antiviral immunity, and 12 members of the TLR family have been identified in mammals.^{67,68)} TLRs are receptive to various components of bacterial cell walls. For example, TLR2 and TLR4 recognize peptidoglycan from gram-positive bacteria and lipopolysaccharide (LPS) from gram-negative bacteria, and subsequently initiate host defense responses against bacteria. In contrast, TLR3 and TLR9 recognize genes of single strand RNA viruses, and DNA viruses such as herpes simplex virus, and initiate the production of antiviral cytokines such as type I interferon (IFN).

Both T and B cells of the adaptive immune system are highly sensitive to ionizing radiation, whereas antigen-presenting cells such as macrophages and DCs are relatively resistant to ionizing radiation-induced cell death, although radiation-induced functional impairments have been reported.²¹⁻²³⁾ We previously showed that X-irradiated human monocytes, which are the precursors of DCs, can differentiate into DCs.⁵²⁾ Furthermore, although DCs derived from X-irradiated monocytes showed low cell surface antigen expression and cytokine responses, and impaired ability to stimulate T cells following exposure to LPS, these cells remained responsive to the mixture of proinflammatory cytokines after X-irradiation.⁶⁹⁾ These observations suggest that X-irradiation affects TLR expression and responsiveness to ligands. However, few studies have assessed the effects of X-irradiation on TLRs, despite the importance of understanding the effects of

ionizing radiation on innate immunity. In the present study, we investigated the effects of ionizing radiation on the expression and ligand responses of TLR2 and TLR4 in THP1 human monocytic cells and THP1-derived macrophages.

2. Materials and methods

Reagents

LPS (*Escherichia coli* 055:B5) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The TLR2/TLR6 agonist FSL-1 was purchased from InvivoGen (San Diego, CA, USA). The fluorescence-labeled monoclonal antibodies (mABs) anti-human TLR2-phycoerythrin (TLR2-PE) and TLR4-PE were purchased from eBioscience (San Diego, CA, USA). Anti-human tumor necrosis factor- α -fluorescein isothiocyanate (TNF- α -FITC) and mouse IgG_{2a}-PE were purchased from Becton Dickinson (San Jose, CA, USA). Mouse IgG₁-FITC was purchased from Beckman Coulter (Fullerton, CA, USA). Phospho-SAPK/JNK (Thr183/Tyr185) mouse mAB, phospho-p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182) rabbit mAB, phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204) rabbit mAB, and Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology Japan, K.K. (Tokyo, Japan). Alexa Fluor[®] 488-conjugated goat anti-mouse IgG was purchased from Invitrogen (Carlsbad, CA, USA). SP600125 and PD98059 were purchased from Sigma-Aldrich, and SB203580 was obtained from Calbiochem (San Diego, CA, USA).

Cell culture

THP1 human acute monocytic leukemia cells were obtained from RIKEN Bio-Resource Center (Tsukuba, Japan). Cells were cultured in RPMI1640 supplemented with 1% penicillin streptomycin (Gibco, Grand Island, NY, USA), and 10% heat-inactivated fetal bovine serum (FBS; Japan Bioserum Co., Ltd., Japan) at 37°C in a humidified atmosphere containing 5% CO₂.

THP1-derived macrophages (macrophage-like cells) were prepared as previously described.⁷⁰⁾ Briefly, THP1 monocytes (2.0×10^5 cells/ml) were plated in 60 mm dishes (IWAKI, Tokyo, Japan) with 4 ml of medium containing 100 ng/ml PMA, and were cultured for 48 h. Differentiation to macrophage-like cells was confirmed by observing morphological changes under a microscope as shown in Fig. 12-[A]. After 48-h culture, the medium containing PMA was replaced with fresh medium not containing PMA, and macrophage-like cells were then used in experiments. Adherent macrophage-like cells were harvested by trypsinization with 0.1% trypsin/ethylenediaminetetraacetate (Gibco). Viable cell numbers were estimated using trypan blue dye exclusion assays.

In vitro X-irradiation

X-irradiation (150 kVp, 20 mA, 0.5 mm Al, and 0.3 mm Cu filters) was performed using an X-ray generator (MBR-1520R-3; Hitachi Medical Corporation, Tokyo, Japan) at a distance of 45 cm from the focus and a dose rate of 1.00–1.04 Gy/min.

Cell surface staining

THP1 monocytes (1.0×10^5 cells/ml) or macrophage-like cells (about 2.0×10^5 cells/ml) were exposed to X-rays and were harvested after 24 h for surface marker analyses. Cells were stained with TLR2-PE or TLR4-PE mAbs for 30 min at 4°C in the dark. Cells were also stained with corresponding PE-conjugated isotype control mouse IgG. After 30 min, the cells were washed with cold PBS (–) and were analyzed using a flow cytometer (Cytomics FC500; Beckman Coulter).

In experiments with MAPK inhibitors, 20 μ M PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), or SP600125 (JNK inhibitor) were added to the culture medium 30 min before X-irradiation.

Measurements of TNF- α concentrations in culture supernatants

THP1 monocytes and macrophage-like cells were exposed to X-rays, and then LPS (1 $\mu\text{g/ml}$) or FSL-1 (50 ng/ml) were added at 24 h after X-irradiation. After further culture for 24 h, culture supernatants were collected for TNF- α measurements using a Quantikine Human TNF- α ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The lowest detectable concentration was 15.6 pg/ml.

Intracellular TNF- α staining

Intracellular TNF- α expression was analyzed using a Fixation and Permeabilization Solution Kit with BD GolgiPlugTM (BD Bioscience). Briefly, THP1 monocytes (1×10^5 cells/ml) or macrophage-like cells (about 2.0×10^5 cells/ml) were exposed to X-rays. At 24 h after X-irradiation, LPS (1 $\mu\text{g/ml}$) or FSL-1 (50 ng/ml) was added to cultures, and the cells were cultured for additional 8 h. GolgiPlug, which inhibits protein transporters, was added to the culture medium at 2 h of 8-h culture. Cells were harvested and fixed using Cytofix/CytopermTM solution (BD Bioscience) for 15 min on ice. After washing twice in Perm/WashTM solution (BD Bioscience), cells were suspended in Perm/WashTM solution and were stained with anti-human TNF- α -FITC mAbs or isotype control at room temperature in the dark. After 30 min, the cells were washed with Perm/WashTM solution and were analyzed using a flow cytometer.

Intracellular phosphoprotein staining

Intracellular phosphorylated MAPK expression was analyzed using a flow cytometer. Briefly, THP1 monocytes (1×10^5 cells/ml) or macrophage-like cells (about 2.0×10^5 cells/ml) were exposed to X-rays. At 1 or 3 h after X-irradiation, cell suspensions were mixed with pre-warmed BD Cytofix buffer (BD Biosciences) and were incubated at 37°C in a water bath for 10 min. After washing with Washing/Staining Solution (PBS containing 1% FBS and 0.09% sodium azide), cells were permeabilized with cold Perm Buffer III (BD Biosciences) for 30 min on ice. After washing twice with

Washing/Staining Solution, cells were suspended in Washing/Staining Solution and were labeled with primary phosphorylated MAPK (p-ERK, p-JNK, and p-p38) antibodies for 30 min at room temperature. Labeled cells were washed twice with Washing/Staining Solution and were then stained with Alexa Fluor[®] 488-conjugated anti-mouse or Alexa Fluor[®] 488-conjugated anti-goat secondary antibodies at room temperature in the dark. As an isotype control, cells were stained with Alexa Fluor[®] 488-conjugated secondary antibodies alone. After 30 min, cells were washed with Washing/Staining Solution and were analyzed using flow cytometry.

Reverse transcription polymerase chain reaction (RT-PCR)

Macrophage-like cells were exposed to X-rays, and LPS (1 µg/ml) was added to culture at 24 h after X-irradiation. After additional 24 h, cells were harvested and total RNA was extracted using an RNeasy MINI kit (Qiagen, Valencia, CA, USA). Total RNA was quantified using a NanoDrop (Thermo, Wilmington, DE, USA) and cDNA templates were synthesized from 1 µg RNA using oligo (dT)₁₂₋₁₈ primers and SuperScript[™] III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Primers for IFN-β were 5'-CTT GTG GCA ATT GAA TGG GAG GC-3' (sense) and 5'-CCA GGC ACA GTG ACT GTA CTC CTT-3' (antisense). β-actin primers were 5'-GGC ACC CAG CAC AAT GAA GA-3' and 5'-GGC ACG AAG GCT CAT CAT TC-3' (antisense). PCR was performed using an AccuPrime[™] Taq DNA Polymerase System (Invitrogen). The reaction conditions for IFN-β were 94°C for 1 min followed by 35 cycles of 95°C for 30 s, 55°C for 2 min, and 72°C for 2 min, and then 72°C, for 10 min. The reaction conditions for β-actin were 94°C for 2 min followed by 18 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then 72°C for 10 min. PCR products were confirmed using electrophoresis on ethidium bromide-stained 1.5% agarose gels. The sizes of the products for IFN-β and β-actin were 370 and 632 bp, respectively.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using Power SYBR[®] Green (Applied Biosystems Inc., Carlsbad, CA, USA) and a StepOnePlus[™] system (Applied Biosystems Inc.) with typical amplification parameters (95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Differences in gene expression relative to non-irradiated controls were determined using ΔC_t values after normalization to the housekeeping gene β -actin. β -actin primer sequences are reported elsewhere.⁷¹⁾ Primers for IFN- β were 5'-GAT TCA TCT AGC ACT GGC TGG-3' (sense) and 5'-CTT CAG GTA ATG CAG AAT CC-3' (antisense).

Statistical analysis

Data are presented as the mean \pm SD. Comparisons of non-irradiated and X-irradiated groups were made using a one-way ANOVA model and the Dunnett's test or Steel's test depending on normality of data distributions. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using Excel 2010 software (Microsoft, USA) with the add-in software Statcel 3.⁷²⁾

3. Results

X-irradiation decreases numbers of viable THP1 monocytes and macrophage-like cells

The effects of ionizing radiation on viable cell numbers of THP1 monocytes and macrophage-like cells were investigated using trypan blue dye exclusion assays. THP1 monocytes retained the ability to grow after exposure to 1 or 2 Gy; however, viable cell numbers were decreased compared with non-irradiated cells (Fig. 12-[B]), and cell growth was not observed after exposure to 5 or 10 Gy. Although macrophage-like cells did not grow after differentiation, and no significant differences in viable cell numbers were observed between non-irradiated and 10 Gy-irradiated cells, cells exposed to lower X-ray doses were significantly fewer than non-irradiated cells (Fig. 12-[C]).

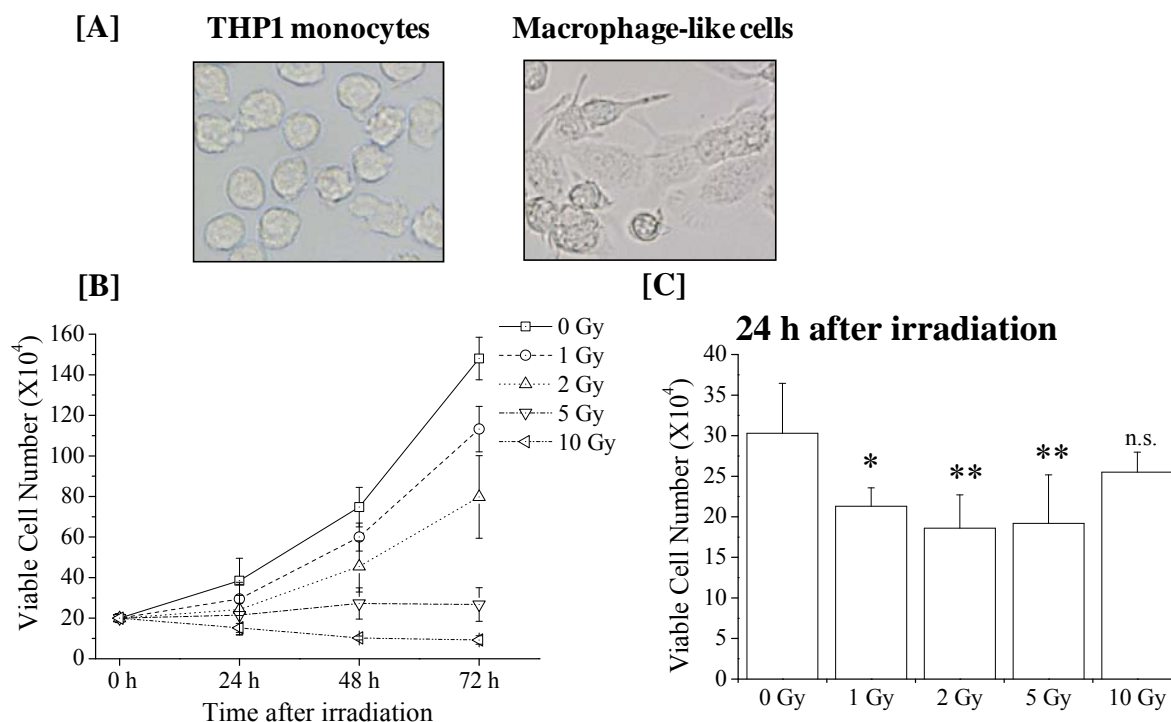


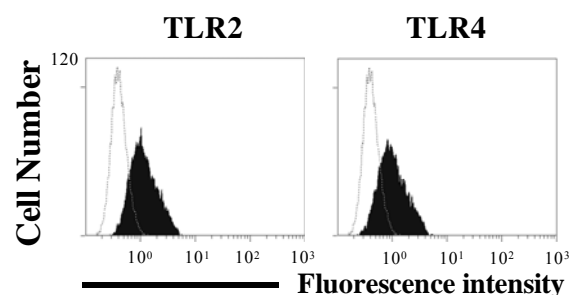
Fig. 12. Cell morphology and viable cell numbers of X-irradiated THP1 monocytes and macrophage-like cells. [A] Morphology of THP1 monocytes [left] and macrophage-like cells [right]. [B] THP1 monocytes were exposed to X-irradiation and were cultured for 24–72 h. Cells were harvested and viable cell numbers were estimated using the trypan blue dye exclusion method. [C] Macrophage-like cells were exposed to X-irradiation and were cultured for 24 h. Cells were harvested and viable cell numbers were estimated using the trypan blue dye exclusion method. Data are presented as the mean \pm SD of four independent experiments; * $p < 0.05$ compared with non-irradiated control; n.s., not significant.

X-irradiation upregulates cell surface TLR expression in THP1 monocytes and enhances responses to ligands

The effect of X-irradiation on TLR2 and TLR4 expression in THP1 monocytes was investigated. As shown in Fig. 13-[A], non-irradiated THP1 expressed both TLR2 and TLR4 on the cell surface. After 24-h exposure of THP1 monocytes to 5 Gy, the expression of TLR2 was increased (Fig. 13-[B]). Similarly, the expression of TLR4 on X-irradiated THP1 monocytes was significantly higher than in non-irradiated cells. Because X-irradiation with 10 Gy dramatically decreased THP1 cell numbers, TLR expression was not analyzed in these cells.

X-irradiation with 5 Gy increased the expression of TLR2 and TLR4. Thus, the effect of X-irradiation on receptor responses to ligands was estimated according to the production of the pro-inflammatory cytokine TNF- α . Although culture supernatants from non-stimulated THP1 monocytes did not contain detectable TNF- α (< 15.6 pg/ml), TNF- α was detectable after stimulation with FSL-1 (ligand for TLR2) or LPS (ligand for TLR4) for 24 h (Fig. 13-[C]). No differences in TNF- α concentrations after stimulation with FSL-1 or LPS were observed between non-irradiated and 5 Gy-irradiated THP1 monocytes (Fig. 13-[C]). However, given that viable cells decreased to 50% and 75% at 24 h and 48 h, respectively, after X-irradiation with 5 Gy (Fig. 12-[B]), the production of TNF- α probably increased at least 2-fold in 5 Gy-irradiated THP1 monocytes compared with non-irradiated cells. Accordingly, analyses of intracellular TNF- α showed increases in percent TNF- α -FITC positive cells and in mean fluorescence intensity of TNF- α -FITC after stimulation of 5 Gy-irradiated cells with FSL-1 or LPS compared with non-irradiated cells (Fig. 13-[D]).

Figure 13 [A]



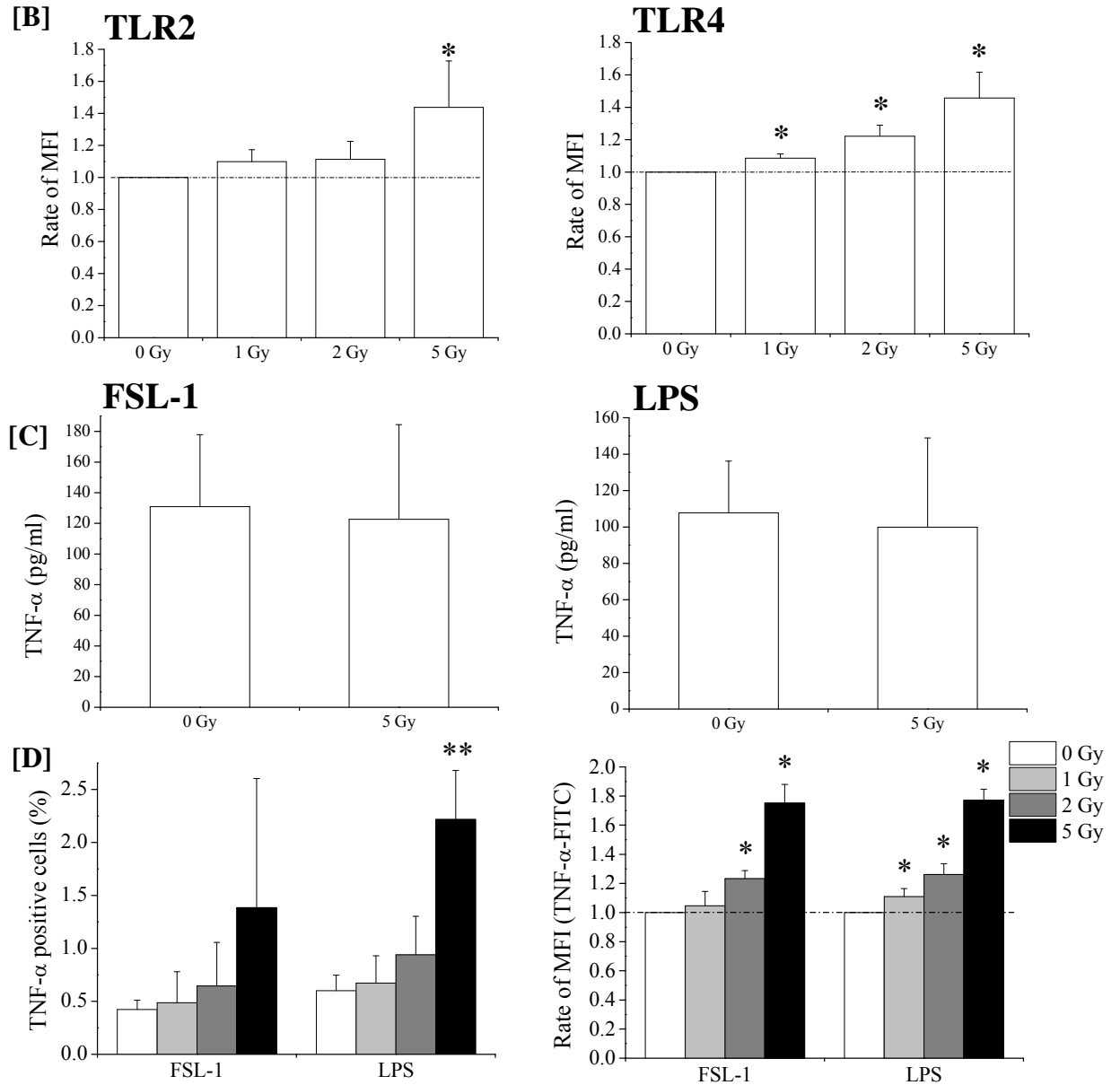


Fig. 13. Effects of X-irradiation on TLR2 and TLR4 expression, and THP1 monocytes responses to ligands. [A] Representative histograms of TLR2 and TLR4 expression in non-irradiated THP1 monocytes; The dotted line indicates the isotype control. [B] Non- or X-irradiated THP1 monocytes were cultured for 24 h, and TLR2 and TLR4 expression was analyzed; MFI, mean fluorescence intensity. Data are the mean \pm SD of four independent experiments. [C] Non- or 5 Gy-irradiated THP1 monocytes were cultured for 24 h, and FSL-1 or LPS were added to culture. After additional 24 h of culture, supernatants were harvested and TNF- α concentrations were measured. Data are presented as the mean \pm SD of four independent experiments. [D] Non- or X-irradiated THP1 monocytes were cultured for 24 h. Cells were then stimulated with FSL-1 or LPS for 8 h and expression of intracellular TNF- α was determined. Data are presented as the mean \pm SD of four independent experiments; * and ** indicate $p < 0.05$ and $p < 0.01$ compared with non-irradiated control, respectively.

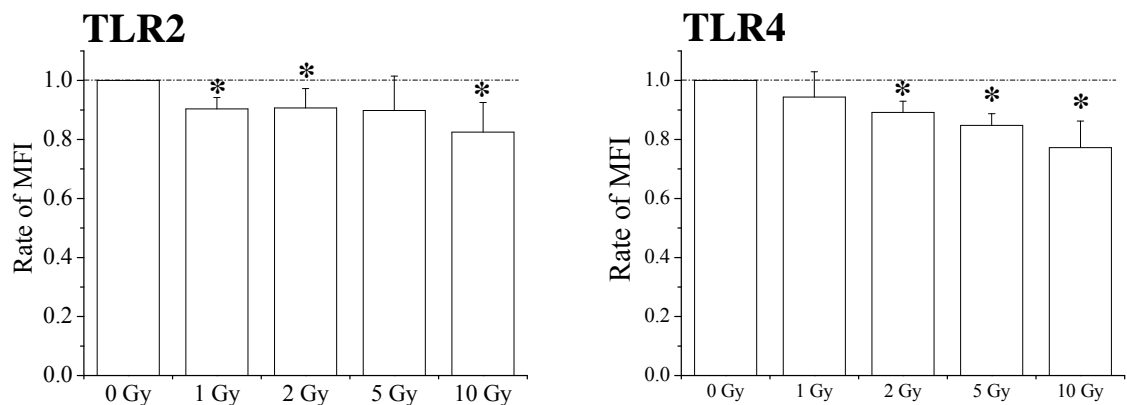
X-irradiation downregulates cell surface TLR expression in macrophage-like cells

The effects of X-irradiation on the expression of TLR2 and TLR4 in macrophage-like cells were investigated. In contrast with THP1 monocytes, X-irradiation decreased cell surface expression of TLR2 and TLR4 in THP1 macrophage-like cells in a dose-dependent manner (Fig. 14-[A]).

Subsequently, TNF- α production by macrophage-like cells was examined after FSL-1 or LPS stimulation. In these experiments, TNF- α concentrations in culture supernatants from non-stimulated macrophage-like cells were ~180 pg/ml (data not shown), and were dramatically increased to ng/ml levels after stimulation with FSL-1 or LPS (Fig. 14-[B]), whereas only pg/ml concentrations were observed in THP1 monocytes (Fig. 13-[C]). Although X-irradiation decreased cell surface TLR expression in macrophage-like cells, the production of TNF- α in these cells was comparable with that in non-irradiated cells (Fig. 14-[B], [C]).

Because LPS induces both TNF- α and antiviral cytokines such as IFN- β ,⁶⁶⁾ the effects of X-irradiation on LPS-induced IFN- β were examined in macrophage-like cells. Although non-stimulated macrophage-like cells did not express IFN- β mRNA (data not shown), IFN- β mRNA expression was observed after LPS stimulation (Fig. 14-[D]), and remained after X-irradiation (Fig. 14-[D]). However, IFN- β expression in X-irradiated macrophage-like cells was lower than that in non-irradiated cells (Fig. 14-[D], [E]). IFN- β was then examined in culture supernatants using ELISA, but was not present at detectable levels (< 50 pg/ml, data not shown).

Figure 14 [A]



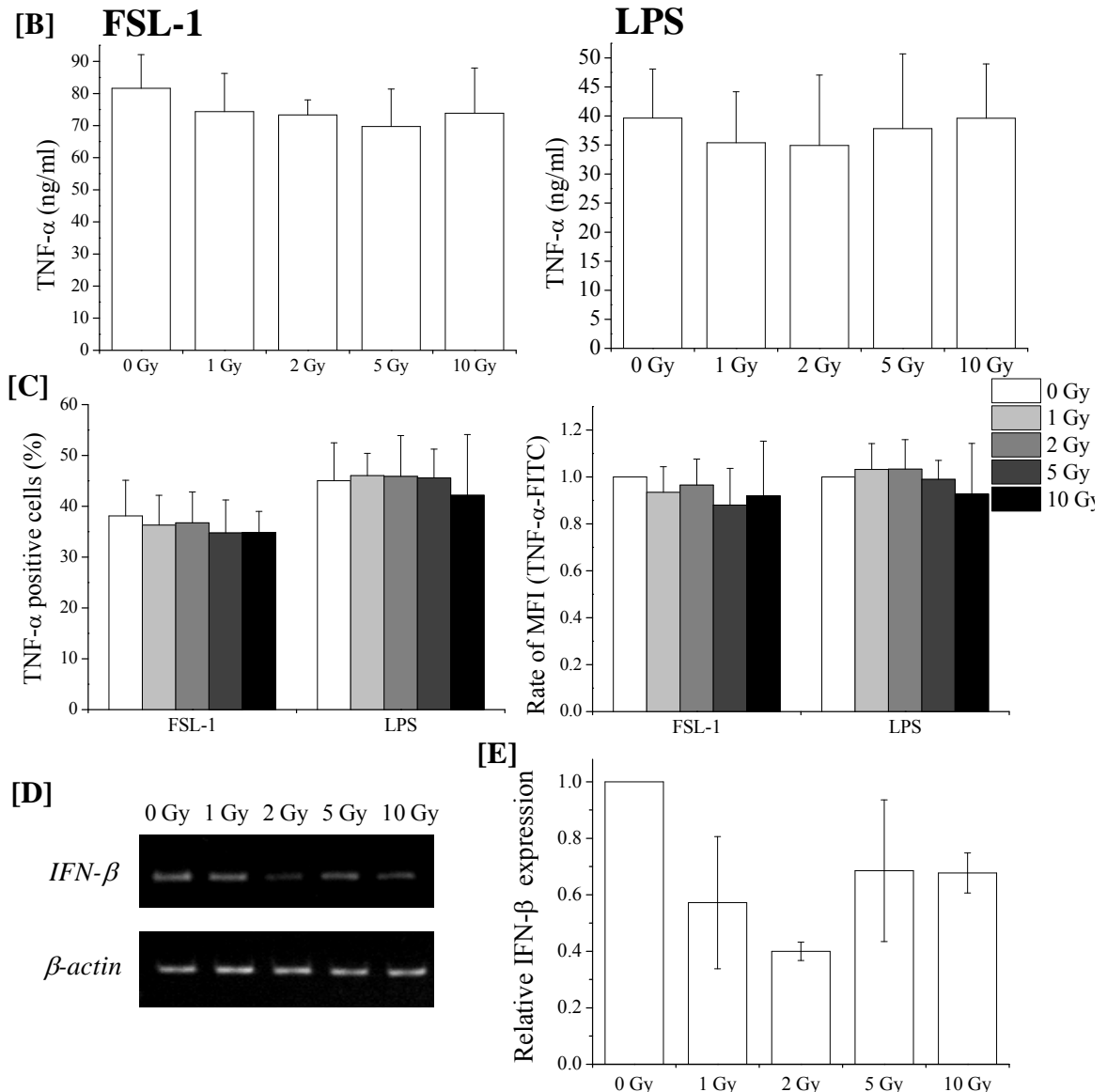


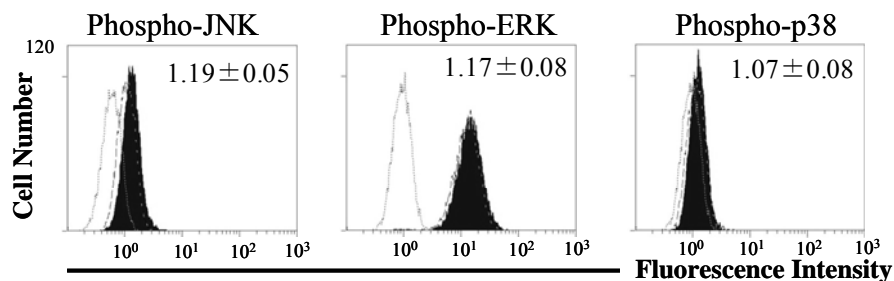
Fig. 14. Effects of X-irradiation on TLR2 and TLR4 expression and response to ligands in macrophage-like cells. [A] Non- or X-irradiated macrophage-like cells were cultured for 24 h and the expression of TLR2 and TLR4 were analyzed. Data are presented as the mean \pm SD of four independent experiments; * $p < 0.05$ compared with non-irradiated control. [B] Non- or X-irradiated macrophage-like cells were cultured for 24 h, and FSL-1 or LPS were added to culture. After additional 24 h of culture, TNF- α concentrations contained in culture supernatants were determined. Data are presented as the mean \pm SD of four independent experiments. [C] Non- or X-irradiated macrophage-like cells were cultured for 24 h, were then stimulated with FSL-1 or LPS for 8 h, and the intracellular TNF- α expression was analyzed. Data are presented as the mean \pm SD of four independent experiments. [D, E] Non- or X-irradiated macrophage-like cells were cultured for 24 h, and LPS was added to culture. After additional 24 h of culture, the IFN- β mRNA expression was determined using RT-PCR [D] and real-time quantitative RT-PCR [E]. Data from real-time quantitative RT-PCR experiments are presented as the mean \pm SD of three independent experiments.

Involvement of MAPK in the regulation of TLR expression after X-irradiation

MAPK signaling pathways are known to regulate TLR expression.^{73,74)} Thus, MAPK-mediated regulation of TLR2 and TLR4 expression was investigated after X-irradiation using MAPK inhibitors. Initially, the relationship between MAPK and TLR2 and TLR4 expression was examined in THP1 monocytes. In these experiments, levels of phosphorylated JNK and ERK in 5 Gy-irradiated cells were ~20% higher than in non-irradiated cells 1 h after X-irradiation (Fig. 15-[A]). Whereas 24-h treatments of non-irradiated THP1 monocytes with the MAPK inhibitors PD98059 (ERK inhibitor) or SP600125 (JNK inhibitor) decreased TLR2 and TLR4 expression, treatment with SB203580 (p38 inhibitor) did not (Fig. 15-[B]). In irradiated cells, treatment with the MAPK inhibitor SP600125 abolished radiation-induced upregulation of TLRs (Fig. 15-[B]), whereas PD98059 partially suppressed radiation-induced TLRs.

Finally, the involvement of MAPK in TLR2 and TLR4 expression was examined in macrophage-like cells. As shown in Fig. 15-[C], treatment with SB203580 or SP600125 decreased TLR2 and TLR4 expression, indicating that JNK and p38 are involved in TLR expression in macrophage-like cells. Phosphorylated JNK levels in 10 Gy-irradiated macrophage-like cells were lower than those in non-irradiated cells at 1 h and 3 h after X-irradiation (Fig. 15-[D]). Moreover, phosphorylated p38 levels were slightly lower in 10 Gy-irradiated cells, but only at 1 h after X-irradiation.

Figure 15 [A]



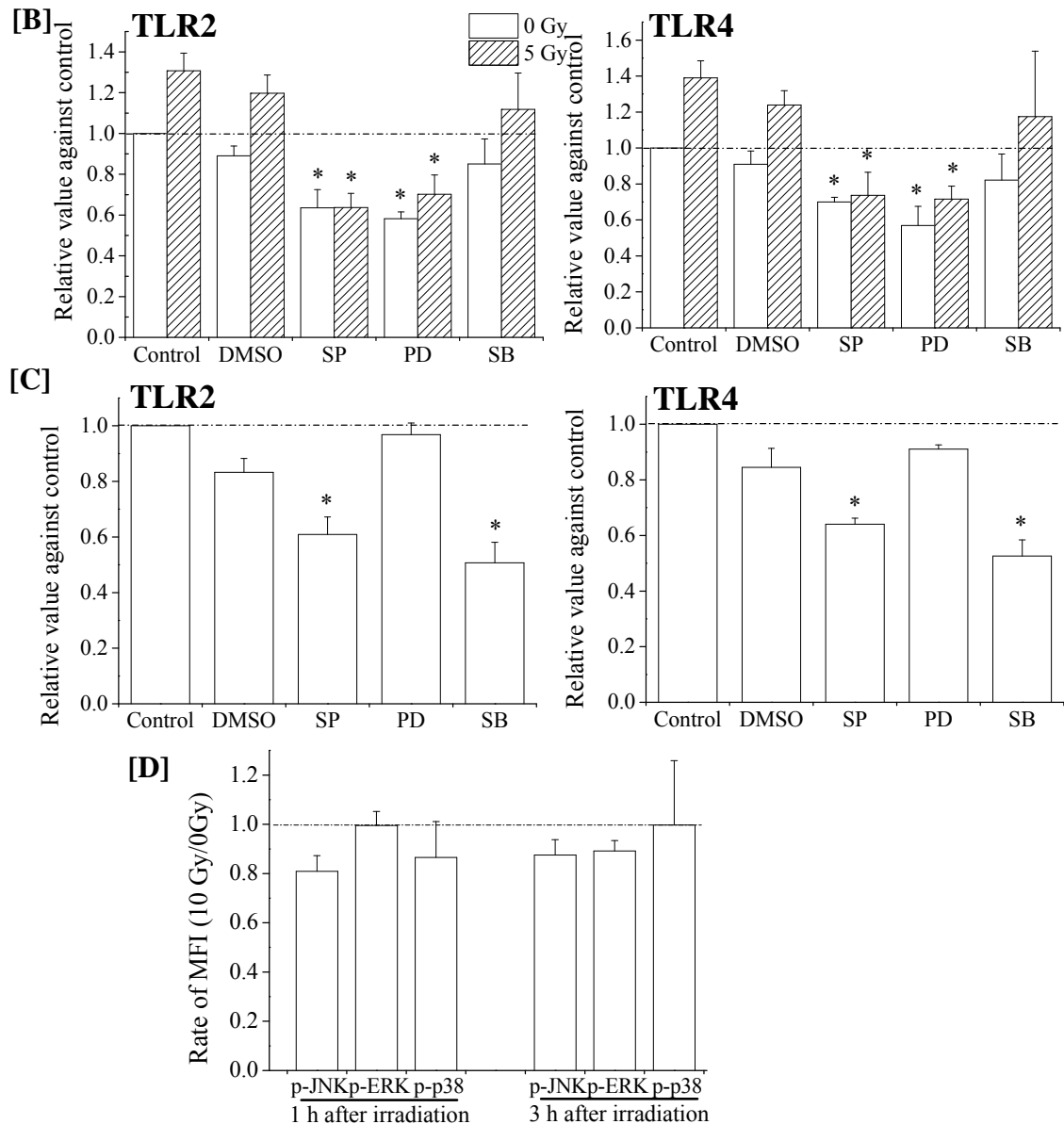


Fig. 15. Effects of MAPK on the regulation of TLR2 and TLR4 expression. [A] Non- or 5 Gy-irradiated THP1 monocytes were cultured for 1 h, and the expression of phosphorylated MAPK was subsequently analyzed. Filled gray bars indicate MAPK expression in 5 Gy-irradiated THP1 monocytes. Dotted and broken lines indicate isotype and non-irradiated controls, respectively. [B] MAPK inhibitors were added to the culture 30 min before X-irradiation. After 24 h of culture, TLR2 and TLR4 expression was analyzed. Inset numbers indicate the relative values of MFI (5 Gy/0 Gy). Data are the mean \pm SD of three independent experiments. [C] TLR2 and TLR4 expression in macrophage-like cells treated with MAPK inhibitors for 24 h; data are the mean \pm SD of three independent experiments. [D] Non- or 10 Gy-irradiated macrophage-like cells were cultured for 1 or 3 h, and expression of phosphorylated MAPK was analyzed. The relative values of MFI (10 Gy/0 Gy) are shown. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$ compared with DMSO treatment. SP600125 (SP); JNK inhibitor, PD98059 (PD); ERK inhibitor, SB203580 (SB); p38 inhibitor.

4. Discussion

The present study demonstrates that X-irradiation affects the expression of TLR2 and TLR4, and that the response to PAMPs depending on the differentiation state of THP1 monocytes. In THP1 monocytes, X-irradiation increased TLR2 and TLR4 expression (Fig. 13-[B]) and enhanced ligand-induced TNF- α production (Fig. 13-[C], [D]); this suggests that X-irradiation enhances the response of THP1 monocytes to PAMPs by upregulating TLRs. However, despite downregulation of TLR expression, production of TNF- α by macrophage-like cells was retained after X-irradiation (Fig. 14-[A–C]). Thus, responses to PAMPs do not necessarily correlate with TLR expression. In a previous report, we showed decreased responses of DCs that were derived from X-irradiated monocytes to LPS compared with non-irradiated cells, despite similar TLR4 expression.⁶⁹⁾ In contrast, LPS-inducible IFN- β expression in macrophage-like cells was attenuated by X-irradiation (Fig. 14-[D], [E]). After LPS recognition, TLR4 activates adaptor proteins such as myeloid differentiation factor 88 (MyD88) and Toll/IL-1R domain-containing adaptor inducing IFN (TRIF).⁴⁸⁾ The MyD88 signaling pathway stimulates production of pro-inflammatory cytokines such as TNF- α , whereas TRIF signaling leads to the production of antiviral cytokines such as IFN- β . Therefore, it is possible that X-irradiation affects TRIF-dependent (but not MyD88-dependent) signaling pathways in macrophage-like cells.

Interestingly, the regulation of TLR2 and TLR4 expression by MAPK varied depending on the cell differentiation state. In brief, JNK and ERK are involved in the TLRs expression on THP1 monocytes (Fig. 15-[B]), whereas JNK and p38 are involved in macrophage-like cells (Fig. 15-[C]). In agreement with previous reports,^{75,76)} phosphorylation of JNK was increased in THP1 monocytes after X-irradiation (Fig. 15-[A]). However, X-irradiation decreased levels of phosphorylated JNK in macrophage-like cells. These results suggest that X-irradiation regulates TLRs through JNK depending on the cell differentiation state. It remains unclear why JNK activation by ionizing radiation depends on the cell differentiation state. However, given that JNK

is sensitive to intracellular redox signaling⁷⁷⁾, and antioxidant enzymes such as superoxide dismutase 2 are involved in radiation resistance,⁷⁸⁾ it is possible that cell differentiation by PMA promotes antioxidant systems, which leads to the differences in both radiation sensitivity and JNK activation after exposure to ionizing radiation (Fig. 12-[B], [C] and Figs. 15-[A], [D]).

Production of TNF- α in X-irradiated THP1 monocytes was higher than in non-irradiated cells after stimulation with PAMPs (Fig. 13-[C], [D]). TLRs recognize both PAMPs and damaged-associated molecular patterns (DAMPs), such as heat shock proteins and high mobility group box 1, which are released from injured tissues and dead cells.⁷⁹⁾ Lambros et al. reported upregulation of several DAMP genes in tissues exposed to ionizing radiation.⁸⁰⁾ Furthermore, death of cancer cells by medical therapy causes the release of DAMPs.⁸¹⁾ Therefore, it is possible that ionizing radiation induces inflammation through the release of DAMPs and through upregulation of TLRs in irradiated immune cells even in the absence of PAMPs. To clarify the involvement of TLRs in radiation-induced inflammation, further studies of the effects of radiation on other TLR-expressing cell types such as endothelial cells⁸²⁾ are required. In conclusion, the present data demonstrate that ionizing radiation affects TLR expression by activating JNK and PAMP responses depending on the differentiation state of cells. These insights into the regulation of TLRs by ionizing radiation elucidate mechanisms of radiation-induced impairment of immune function and radiation-induced inflammation.

Conclusions

The present studies demonstrated that human peripheral blood monocytes exposed to ionizing radiation can differentiate into DCs, but there is a tendency that X-irradiation causes an impairment of the function of DCs. Especially, the DCs derived from the X-irradiated monocytes showed a lower response to LPS as compared with the DCs derived from the nonirradiated monocytes, whereas they largely retained the response to inflammatory cytokine mixture. Furthermore, the study using monocytic cell line demonstrated that ionizing radiation affects TLR expression and PAMP responses, which is indispensable for immune defense against pathogens, by activating JNK and depending on the differentiation state of cells. We believe that further studies regarding the mechanisms of impairment of DCs derived from the X-irradiated monocytes and the regulation of TLRs by ionizing radiation leads to the establishment of effective cancer therapy combined with radiation and immunotherapy as well as the recovery of immune system after radiation exposure.

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要 旨

樹状細胞の分化誘導および Toll 様受容体に及ぼす放射線の影響

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樹状細胞は抗原提示細胞の一つで、自然免疫と獲得免疫を繋ぐ免疫システムにおいて必要不可欠な細胞である。これまでの報告により、分化した樹状細胞は放射線による細胞死に対して抵抗性を示すが、放射線曝露樹状細胞では機能低下が起こることが明らかとなっているもの、樹状細胞の分化誘導における放射線の影響については不明である。

本研究では、樹状細胞の前駆細胞のヒト末梢血単球に着目し、ヒト末梢血単球由来樹状細胞の分化誘導における放射線の影響を検討した。ヒト末梢血由来単球に X 線 5 Gy を照射し、サイトカイン存在下で未熟樹状細胞へ分化誘導を行ったところ、未熟樹状細胞に発現する細胞表面発現抗原の発現や未熟樹状細胞の特徴である貪食能が確認された。また、腫瘍壊死因子 α を用いて成熟樹状細胞へ誘導したところ、成熟マーカーである CD83 などの発現が観察されたが、成熟樹状細胞の主要な機能である T 細胞刺激能力などが 5 Gy 照射群で低下していた。次に、グラム陰性細菌細胞壁構成成分のリポ多糖または炎症性サイトカインミックスで刺激し成熟樹状細胞へ誘導を行ったところ、リポ多糖刺激では、X 線照射によってサイトカイン産生及び T 細胞刺激能力が著しく低下した一方で、炎症性サイトカインミックス刺激では X 線照射による低下は認められなかった。

リポ多糖などの病原体関連分子はパターン認識受容体である Toll 様受容体(以下、TLR)によって認識されるため、最後に TLR に及ぼす放射線の影響をヒト単球系細胞 THP1 を用いて検討した。その結果、未分化 THP1 細胞では X 線曝露後に TLR2 及び TLR4 の発現増強が起きるとともに、それぞれのアゴニスト誘導性の炎症性

サイトカイン産生が増加した。一方で、THP1 由来マクロファージでは X 線照射により TLR2 及び TLR4 の発現が低下したが、アゴニスト誘導性の炎症性サイトカイン産生は非照射細胞と同程度であった。

本研究結果より、放射線曝露単球は樹状細胞に分化できるものの、一部の機能低下が起きることが示された。また、X 線曝露ヒト単球由来成熟樹状細胞の機能障害は成熟刺激に依存し、放射線によって外因性因子のリポ多糖に対する応答性が低下するが明らかとなった。さらに、TLR に及ぼす放射線の影響は細胞分化に依存することが明らかとなった。

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