

Antitumor effects of NK cells expanded by activation pre-processing of autologous feeder cells before irradiation in colorectal cancer

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Abstract. Natural killer (NK) cells play a crucial role in early immune defenses against transformed cells and are used in the therapeutic management of cancer. However, it is difficult to sufficiently obtain high purity activated NK cells for clinical application. The function of NK cells is dependent on the balance of activating and inhibitory signals. Strong and diverse stimuli are required to increase the function of NK cells. Radiotherapy modulates the expression of various immunomodulatory molecules that recruit and activate NK cells. NK cell-mediated antibody-dependent cellular cytotoxicity is one of the most potent cytotoxic effects of NK cells against target cancer cells. To generate activated and irradiated autologous peripheral blood mononuclear cells (PBMCs), cytokine and monoclonal antibody stimulation followed by ionizing radiation was performed in the present study. The expanded NK cells were cultured for 21 days using activated/irradiated autologous PBMCs. Colorectal cancer cells (SW480 and HT-29) were used to analyze the expression of NK group 2D ligands and EGFR by radiation. The cytotoxicity of radiation plus NK cell-based targeted therapy against colorectal cancer cell lines was analyzed using flow cytometry. Activated and irradiated PBMCs exhibited significantly increased expression

of various activating ligands that stimulated NK cells. In total, >10,000-fold high-purity activated NK cells were obtained, with negligible T-cell contamination. To confirm the anti-tumor activity of the NK cells expanded by this method, the expanded NK cells were treated with cetuximab, radiotherapy, or a combination of cetuximab and radiotherapy in the presence of human colorectal cancer cells. Expanded NK cells were effective at targeting human colorectal cancer cells, particularly when combined with cetuximab and radiotherapy. Thus, in the present study, a novel method for high-purity activated NK cell expansion was developed using activated and irradiated PBMCs. In addition, combined radiotherapy and antibody-based immunotherapy with expanded NK cells may be an effective strategy to enhance the efficiency of treatment against colorectal cancer.

Introduction

Natural killer (NK) cells are powerful cytotoxic lymphocytes that play a vital role in the innate immune response by eliminating abnormal cells without relying on specific antigens (1,2). The function and specificity of NK cells are determined by the binding of activating and inhibitory receptors that bind to various ligands on the surface of the target cells (2,3). NK cells are specifically sensitive to cancer or transformed cells that exhibit reduced or absent expression of major histocompatibility complex (MHC) class I molecules. By contrast, NK cells have low sensitivity to cancer cells with high expression of MHC class I molecules (2,4). Thus, a stronger additional activation signal is needed to overcome this shortcoming of NK cells.

Ionizing radiation (IR) gives rise to systemic antitumor immune responses as well as local antitumor effects by expressing a variety of immunomodulatory molecules that recruit and stimulate immune cells, such as macrophages, and dendritic, T, and NK cells (5,6). IR induces the expression of various immune stimulatory molecules, such as MHC class I molecules, NK group 2D (NKG2D) ligands, co-stimulatory molecules, Fas/CD95, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (5-8). In particular, NKG2D

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ligands are important key factors for increasing the sensitivity of NK cells to cancer cells. Radiation-induced NKG2D ligands showed different expression patterns for a variety of cancer cells (9-11).

NK cells express low-affinity Fc immunoglobulin G (IgG) receptor (FcγRIII/CD16), which triggers antibody-dependent cellular cytotoxicity (ADCC). ADCC is one of the major immune effector mechanisms responsible for the efficacy of antibody-based cancer therapies (12).

EGFR is overexpressed in various types of malignant cells present in colorectal, head and neck, lung, and pancreatic cancer; such types of cancer have a poor prognosis (13-16). Cetuximab (Erbix) is a chimeric IgG1 monoclonal antibody (mAb) that binds to EGFR and has been approved by the Food and Drug Administration for treating patients with metastatic colorectal cancer (17). However, the treatment efficacy of patients with metastatic colorectal cancer was limited when cetuximab alone was used (18). Thus, the efficacy of cetuximab could be enhanced by NK-mediated immunotherapy to provoke ADCC in antibody-coated target cells.

NK cells have been investigated in a variety of therapeutic strategies for the management of cancer (19,20). However, it is necessary to obtain a sufficient number of cells with high purity for the therapeutic use of NK cells. Previous studies have reported methods for large-scale NK cell expansion using various cancer cell-derived feeder cells that activate NK cells through cell-to-cell contact (21-25). Our previous study reported a method for large-scale NK cell expansion by combining an anti-CD16 mAb and autologous PBMCs irradiated with 25 Gy (26). This method provided an appropriate environment for activating and expanding NK cells, and effectively inhibited the proliferation of T cells. In the present study, a novel method to enhance the expression levels of various activating ligands that stimulated the sensitivity of NK cells to PBMCs was developed to more effectively expand NK cells. This new method expands high-purity activated NK cells >10,000-fold and is accompanied by limited contamination from other cells.

Several studies on the combination of targeted antibody therapy and NK cells have been reported (27-29), but the combination treatment using radiation and antibody therapy together with NK cells has not been studied to the best of the authors' knowledge. The present study investigated the effects of these combinations on SW480 and HT-29 human colorectal cancer cells using expanded NK cells. Radiotherapy and targeted antibody therapy were simultaneously applied to highly cytotoxic NK cells expanded *in vitro* to treat the target cancer more effectively. Taken together, it was demonstrated that NK cells were efficiently expanded *in vitro* by activated/irradiated PBMCs, and this multimodal approach more effectively eliminated target cancer cells.

Materials and methods

Human cancer cell lines. Two human colorectal cancer cell lines, SW480 (cat. no. CCL-288) and HT29 (cat. no. HTB-38) were obtained from American Type Culture Collection and characterized by STR profiling. All cell lines were cultured in RPMI 1640 medium (Welgene, Inc.) supplemented with 10% FBS (Biowest) and antibiotic-antimycotic (Thermo Fisher

Scientific, Inc.) and maintained at 37°C in a humidified atmosphere supplied with 5% CO₂ air.

Generation of activated and irradiated PBMCs. Experiments using human blood were approved (approval no. D-2002-032-002) by the Institutional Review Board (IRB) of Dongnam Institute of Radiological & Medical Sciences (Jangan-eup, South Korea), and written informed consent was obtained from all donors prior to participation in the present study. Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors, and the buffy coat layer was separated by density gradient centrifugation using Lymphoprep™ reagent, according to the manufacturer's protocol (Stemcell Technologies, Inc.; cat. no. 07801). The buffy coat layer was harvested and washed 3 times with normal saline (Dai Han Pharm. Co., Ltd.). The isolated PBMCs were incubated for ≥30 min with or without 500 ng/ml anti-human CD3 (GMP CD3 pure; Miltenyi Biotec GmbH; cat. no. 170-076-116) mAb, 1,000 U/ml recombinant human (rh) IFN-γ (R&D Systems, Inc.; cat. no. 285-GMP-100), and 1,000 U/ml rhIL-2 (Proleukin; Novartis). Activated PBMCs were washed 3 times with normal saline, and 1 ml NK culture medium was added. The cells were irradiated with or without a dose of 25 Gy in a blood irradiator (Eckert & Ziegler), and cultured for 0, 24, 48, and 72 h. The cells were incubated with antibodies against human CD48-fluorescein isothiocyanate (FITC; 1:50; BD Pharmigen; BD Biosciences; cat. no. 555759), CD112-phycoerythrin (PE; 1:50; BD Pharmigen; BD Biosciences; cat. no. 551057), CD155-PE (1:50; R&D Systems, Inc.; cat. no. FAB25301P) and NKG2D ligands [including MHC class I polypeptide-related sequence A (MICA)-PE (1:50; R&D Systems Inc.; cat. no. FAB1300P), MHC class I polypeptide-related sequence B (MICB)-PE (1:50; R&D Systems Inc.; cat. no. FAB1599P), UL16 binding protein (ULBP)-1-PE (1:50; R&D Systems Inc.; cat. no. FAB1380P), ULBP-2/5/6-PE (1:50; R&D Systems Inc.; cat. no. FAB1298P) and ULBP-3-PE (1:50; R&D Systems Inc.; cat. no. FAB1517P)] for 20 min in the dark at room temperature. The cells were also incubated with each isotype control immunoglobulin (Ig)M-FITC (1:50; BD Pharmigen; BD Biosciences; cat. no. 555583), IgG1-PE (1:50; BD Pharmigen; BD Biosciences; cat. no. 555749, 1:50; R&D Systems Inc.; cat. no. IC002P), IgG2b-PE (1:50; R&D Systems Inc.; cat. no. IC0041P), and IgG2a-PE (1:50; R&D Systems Inc.; cat. no. IC003P) for 20 min in the dark at room temperature and evaluated by flow cytometry (FCM) on an FC 500 system (Beckman Coulter, Inc.). Data were analyzed using CXP version 2.2 software (Beckman Coulter, Inc.).

Analysis of the expression levels of NKG2D ligands, MHC class I, and EGFR. SW480 and HT-29 cells were cultured in RPMI 1640 complete medium and maintained at 37°C in a humidified incubator supplied with 5% CO₂ air. The cells were harvested at 0, 24, or 48 h after irradiation at 4 or 8 Gy. The cells were incubated with anti-human EGFR (10 μg/ml; Thermo Fisher Scientific, Inc.; cat. no. MA5-13070), human leukocyte antigen (HLA)-ABC-PE (1:50; BD Pharmigen, BD Biosciences; cat. no. 555553), HLA-E-PE (1:50; BD Pharmigen; BD Biosciences; cat. no. 566921) and NKG2D ligands (MICA; 2.5 μg/ml; R&D Systems Inc.; cat. no. MAB1300, MICB; 2.5 μg/ml; R&D Systems Inc.; cat. no. MAB1599, ULBP-1;

2.5 $\mu\text{g/ml}$; R&D Systems Inc.; cat. no. MAB1380), ULBP-2/5/6 (2.5 $\mu\text{g/ml}$; R&D Systems Inc.; cat. no. MAB1298), or ULBP-3; 2.5 $\mu\text{g/ml}$; R&D Systems Inc.; cat. no. MAB1517) antibodies for 20 min in the dark at room temperature. EGFR and NKG2D ligand detection was performed with a secondary PE-goat anti-mouse IgG (1:500; multiple adsorption; BD Pharmigen; BD Biosciences; cat. no. 550589). The cells were also incubated with each isotype control IgG1-unconjugated (10 $\mu\text{g/ml}$; Thermo Fisher Scientific, Inc.; cat. no. 14-4714-82), IgG1-PE (1:50; BD Pharmigen; BD Biosciences; cat. no. 555749), IgG2B-unconjugated (2.5 $\mu\text{g/ml}$; R&D Systems Inc.; cat. no. MAB0041), and IgG2A-unconjugated (2.5 $\mu\text{g/ml}$; R&D Systems Inc.; cat. no. MAB003) for 20 min in the dark at room temperature and were analyzed by FCM.

NK cell isolation and expansion. NK cells were isolated from whole blood by negative selection (untouched cell isolation) using the EasySep™ Direct Human NK Cell Isolation Kit (Stemcell Technologies, Inc.) according to the manufacturer's instructions. Alternatively, NK cells could be isolated from various kits (NK Cell Isolation kit, Miltenyi Biotec GmbH; cat. no. 130-092-657) based on untouched cell isolation. The purity of the isolated NK cells was evaluated by FCM using anti-human CD3-FITC (1:100; Beckman Coulter, Inc.; cat. no. A07746) and CD56-PE-cyanine 5 (1:100; Beckman Coulter, Inc.; cat. no. A07789) mAbs. The cells were also incubated with each isotype control IgG1-FITC (1:100; Beckman Coulter, Inc.; cat. no. A07795), and IgG1-PC5 (1:100; Beckman Coulter, Inc.; cat. no. A07798) for 20 min in the dark at room temperature. Isolated NK cells (1×10^5 cells/ml) alongside activated and irradiated autologous PBMCs (2×10^6 cells/ml) were co-cultured in coated plates with 5 $\mu\text{g/ml}$ anti-human CD16 mAb (eBioscience; Thermo Fisher Scientific, Inc.; cat. no. 16-0167-82). NK cells were cultured in the presence of 500 IU/ml rhIL-2 and 5% human serum (Biowest) in CellGenix Serum-free Good Manufacturing PracticeMedia (CellGenix; Sartorius; cat. no. 20801-0500). On days 7-8, the cells were transferred to a larger culture flask containing Lymphocyte Growth Medium 3 (Lonza Group Ltd.; cat. no. CC-3211) containing 500 IU/ml rhIL-2 and 5% human serum. Fresh culture medium was added every 2 to 3 days for 18-21 days. NK cells were manufactured under GMP conditions.

NK cell phenotype analysis. NK cells were incubated with anti-human CD3-FITC (1:100; Beckman Coulter, Inc.; cat. no. A07746), anti-human CD16-PE (1:100; Beckman Coulter, Inc.; cat. no. A07766), anti-human CD56-PE-cyanine5 (1:100; Beckman Coulter, Inc.; cat. no. A07789), anti-human CD314-PE (1:100; Beckman Coulter, Inc.; cat. no. A08934), anti-human CD226 [DNAX accessory molecule (DNAM)-1]-FITC (1:50; BD Pharmigen; BD Biosciences; cat. no. 559788), or anti-human CD244 (2B4)-FITC (1:50; BD Pharmigen; BD Biosciences; cat. no. 550815) for 20 min in the dark at room temperature. The cells were also incubated with each isotype control: IgG1-FITC (1:100; Beckman Coulter, Inc.; cat. no. A07795), IgG1-PE (1:100; Beckman Coulter, Inc.; cat. no. A07796), IgG1-PE-cyanine5 (1:100; Beckman Coulter, Inc.; cat. no. A07798), IgG1-FITC (1:50; BD Pharmigen; BD Biosciences; cat. no. 555748), and IgG2a-FITC (1:50; BD Pharmigen; BD Biosciences;

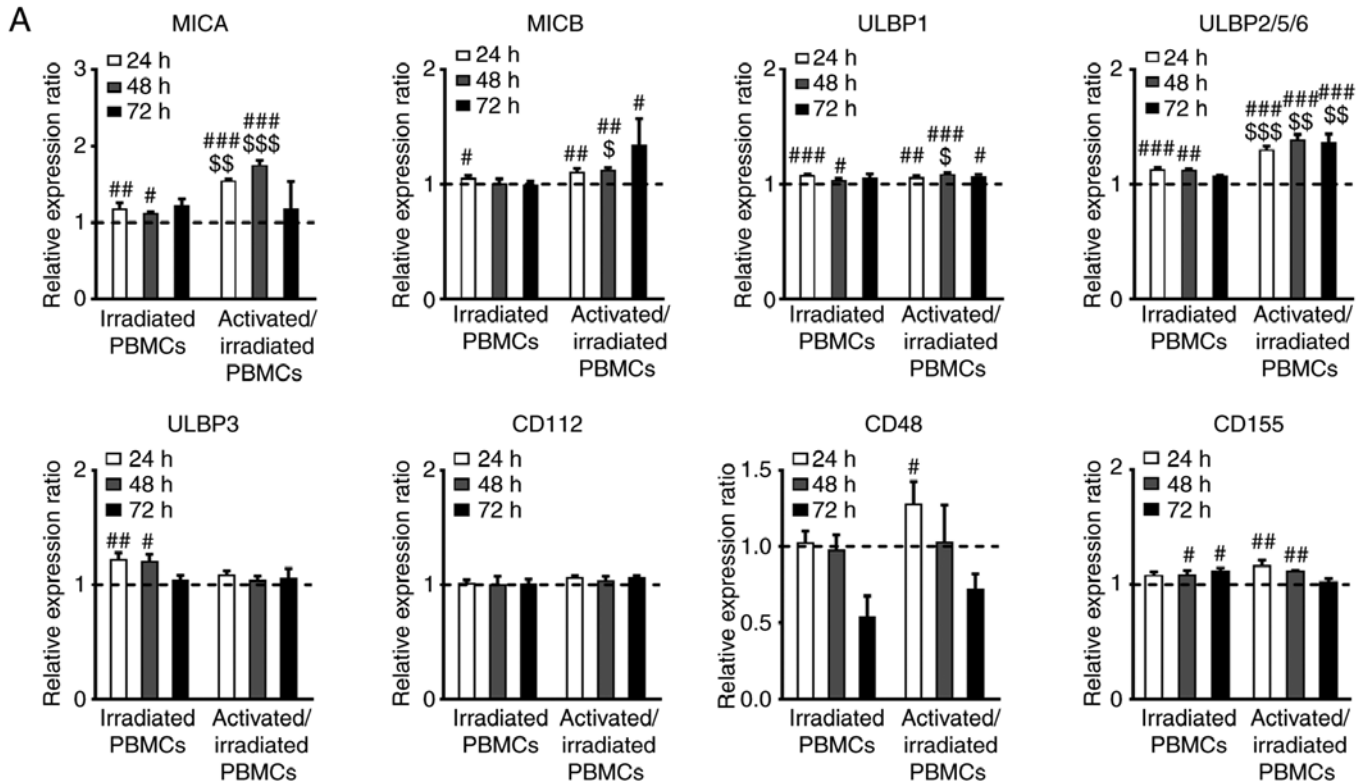
cat. no. 555573) for 20 min in the dark at room temperature and evaluated by FCM.

NK cell-mediated cytotoxicity assay. SW480 and HT-29 cells (target cells) were seeded at 5×10^5 cells/dish 2 days before the cytotoxicity experiments. One day after seeding, the cells were irradiated with or without a dose of 8 Gy in a blood irradiator and cultured for 24 h. After 24 h, the cells were co-cultured with or without 10 $\mu\text{g/ml}$ cetuximab (Merck KGaA; Erbitux injection 5 mg/ml) for 30 min at 37°C. The cells were washed with PBS three times and stained with carboxyfluorescein succinimidyl ester (CFSE)-FITC (eBioscience; Thermo Fisher Scientific, Inc.; cat. no. 65-0850-84) at a final concentration of 5 μM for 10 min at 37°C. After labeling, the reaction was stopped with FBS, and the cells were washed 3 times with normal saline. NK and CFSE-labeled target cells were seeded into round-bottomed 96-well plates with different effector-to-target cell number ratios (10:1, 5:1, 2.5:1 and 1:1) and incubated for 4 h. Subsequently, the cells were transferred to a round bottom 5-ml tube. Propidium iodide (MilliporeSigma) was added to a final concentration of 2 $\mu\text{g/ml}$ for dead cell DNA labeling, and the dead cells were then measured by FCM. The following groups were established: NK alone (NK + SW480 or HT-29); IR + NK (NK + irradiated SW480 or HT-29); and cetuximab + IR + NK (NK + irradiated SW480 or HT-29 + cetuximab).

Statistical analysis. Statistical analysis was performed in SPSS version 18.0 (IBM Corp.) and GraphPad Prism version 6.0 (GraphPad Software, Inc.). Data are presented as the mean \pm standard deviation of 3 repeats. A paired Student's t-test was used to compare the expression of molecules on the cell surface before and after irradiation or activation plus irradiation. For comparisons between the two groups, an unpaired Student's t-test was used, and a one-way ANOVA followed by a Tukey's post hoc test was used to compare multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

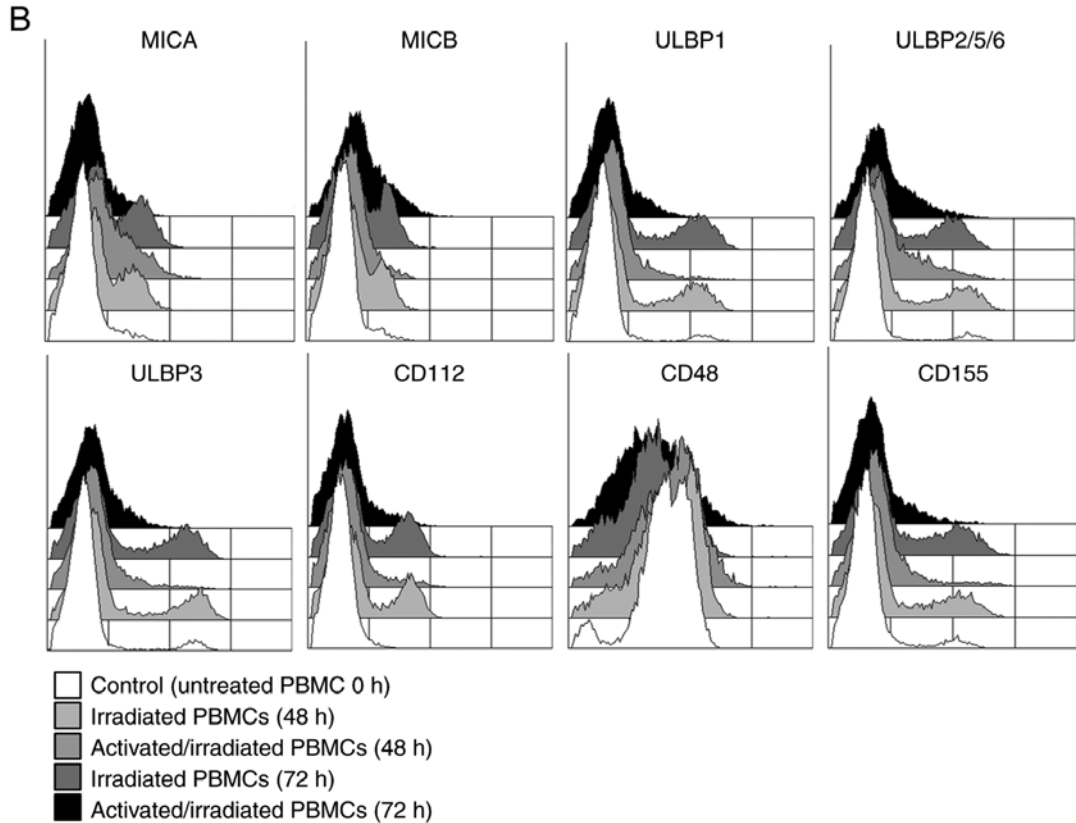
Results

A combination of anti-CD3 mAb, IFN- γ , and IL-2 enhances radiation-induced activating ligand expression in human PBMCs. Our previous study developed a large-scale NK cell expansion method using irradiated autologous PBMCs (26). In the present study, PBMCs isolated from healthy donors were activated by treatment with anti-CD3 mAb, IFN- γ , and IL-2, and then irradiated with 25 Gy. Radiation alone and activated/irradiated PBMCs were cultured for 0, 24, 48, or 72 h, and then the expression of NKG2D, 2B4, and DNAM-1 ligands were analyzed by FCM at each time point. The cell surface expression levels were quantified using median fluorescence intensities (MFIs). Relative expression ratios were calculated by dividing the MFI of the 24, 48, and 72 h samples by that of the untreated PBMCs (0 h). As shown in Fig. 1, the various activating ligands that stimulated the sensitivity of NK cells showed different patterns depending on the donor and time. Irradiated PBMCs exhibited significantly increased expression levels of MICA, MICB, ULBP1, ULBP2/5/6, ULBP3, and CD155 compared with those of untreated PBMCs. Activated/irradiated PBMCs also exhibited significantly



*PBMCs+IR are named irradiated PBMCs.

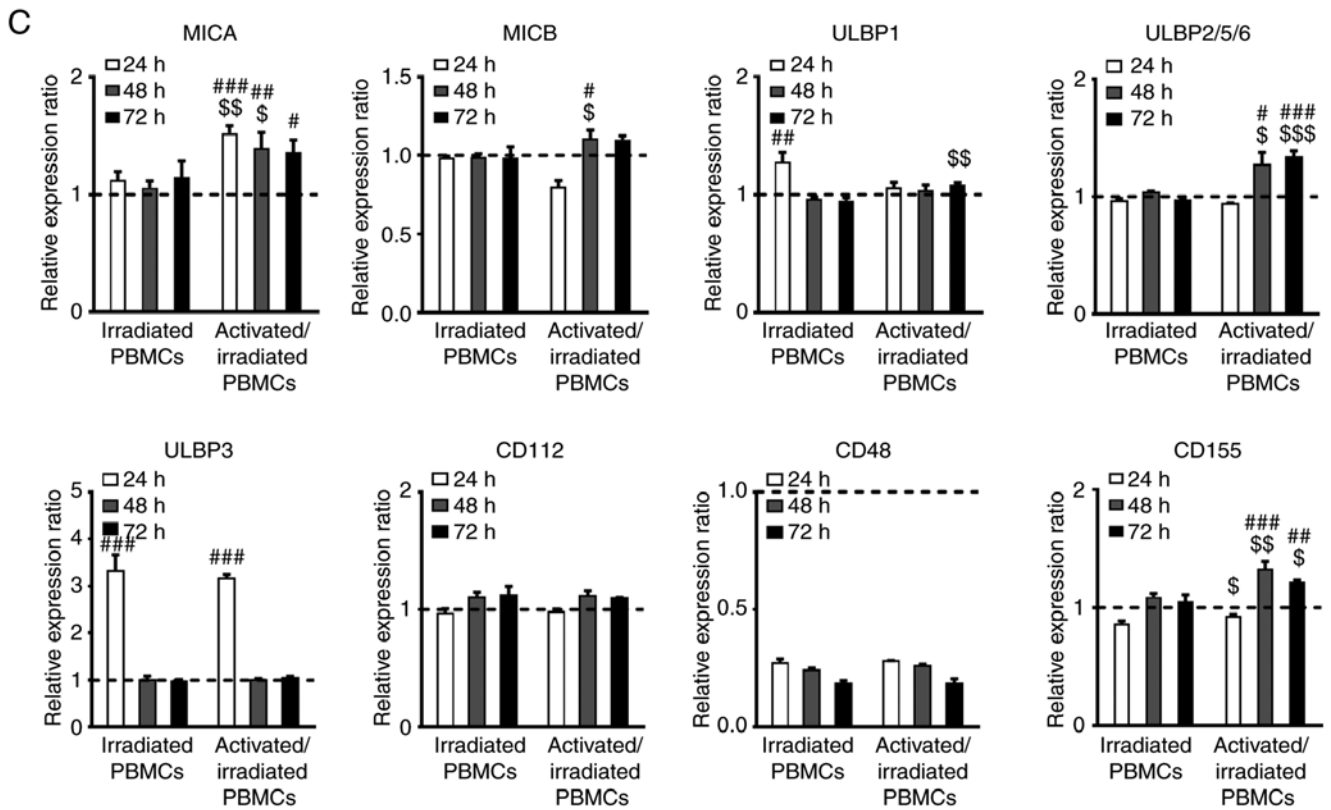
*PBMCs+IR+aCD3 mAb+rhIFN- γ +rhIL-2 are named Activated/irradiated PBMCs.



*PBMCs+IR are named irradiated PBMCs.

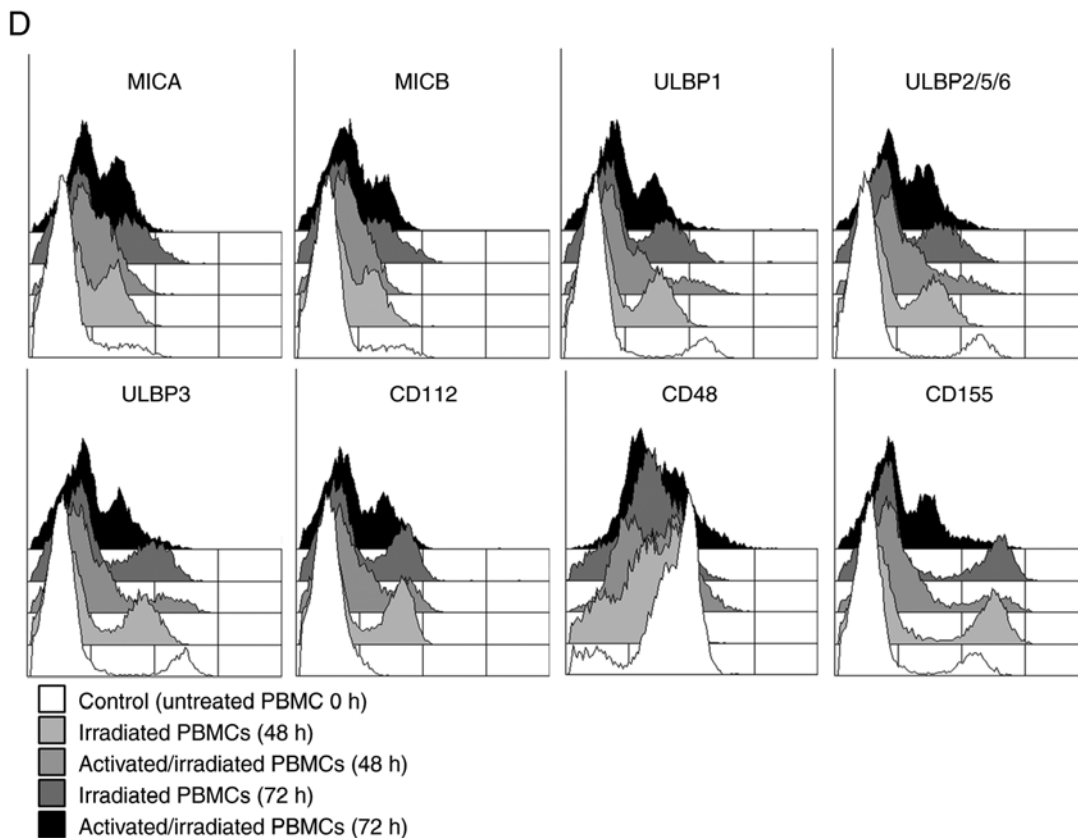
*PBMCs+IR+aCD3 mAb+rhIFN- γ +rhIL-2 are named Activated/irradiated PBMCs.

Figure 1. Continued.



*PBMCs+IR are named irradiated PBMCs.

*PBMCs+IR+aCD3 mAb+rhIFN-r+rhIL-2 are named Activated/irradiated PBMCs.



*PBMCs+IR are named irradiated PBMCs.

*PBMCs+IR+aCD3 mAb+rhIFN-r+rhIL-2 are named Activated/irradiated PBMCs.

Figure 1. Continued.

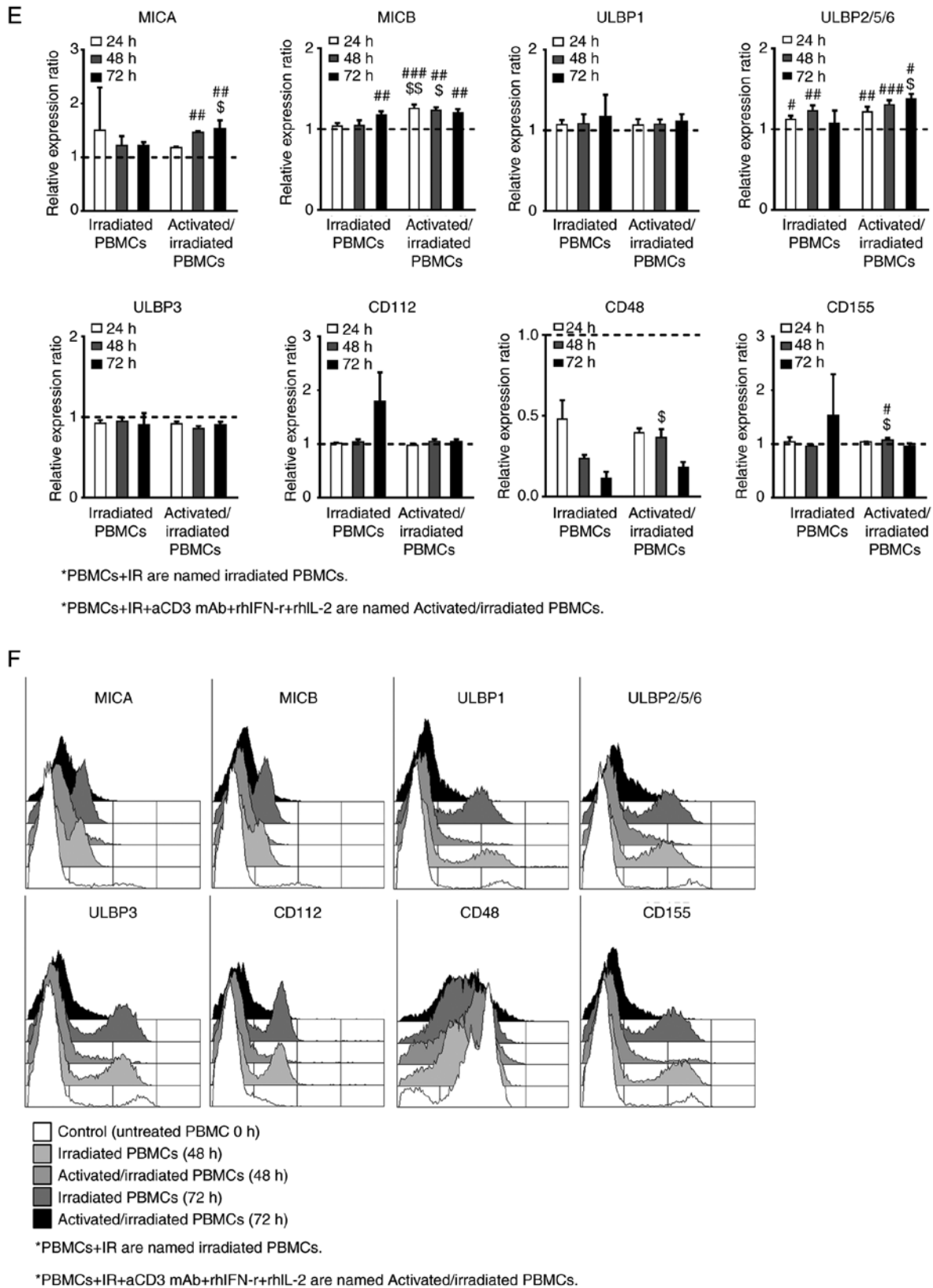


Figure 1. Combination of anti-CD3 mAb, IFN-r, and IL-2 with radiation further promotes the expression levels of activating ligands in human PBMCs. PBMCs were cultured with or without anti-CD3 mAb, IFN-r, and IL-2 and then irradiated with 25 Gy. The cells were cultured for 0, 24, 48, or 72 h. CD48, CD112, CD155, and natural killer group 2D ligands (MICA, MICB, ULBP-1, ULBP-2/5/6, and ULBP-3) were analyzed by flow cytometry. (A, C, and E) Ratios of MFIs obtained from irradiated PBMCs and activated/irradiated PBMCs. Relative expression ratios were calculated by dividing the MFI of irradiated PBMCs (24, 48, and 72 h) or activated/irradiated PBMCs (24, 48, and 72 h) by that of untreated PBMCs (0 h). (B, D, and F) Representative flow cytometry histograms of each donor [white, untreated PBMCs (0 h); bright gray, irradiated PBMCs (48 h); gray, activated/irradiated PBMCs (48 h); dark gray, irradiated PBMCs (72 h); black, activated/irradiated PBMCs (72 h)]. The data are presented as the mean \pm SD of 3 donors. Statistical significance was determined using paired and unpaired Student's t-test. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ [untreated PBMCs (0 h) vs. 24, 48, or 72 h irradiated or activated/irradiated PBMCs]. $^{\$}P < 0.05$, $^{ss}P < 0.005$, $^{sss}P < 0.0005$ (24, 48, and 72 h irradiated PBMCs vs. 24, 48, and 72 h activated/irradiated PBMCs). mAb, monoclonal antibody; r, recombinant; PBMCs, peripheral blood mononuclear cells; MFI, median fluorescence intensity; MIC, MHC class I polypeptide-related sequence; ULBP, UL16 binding protein.

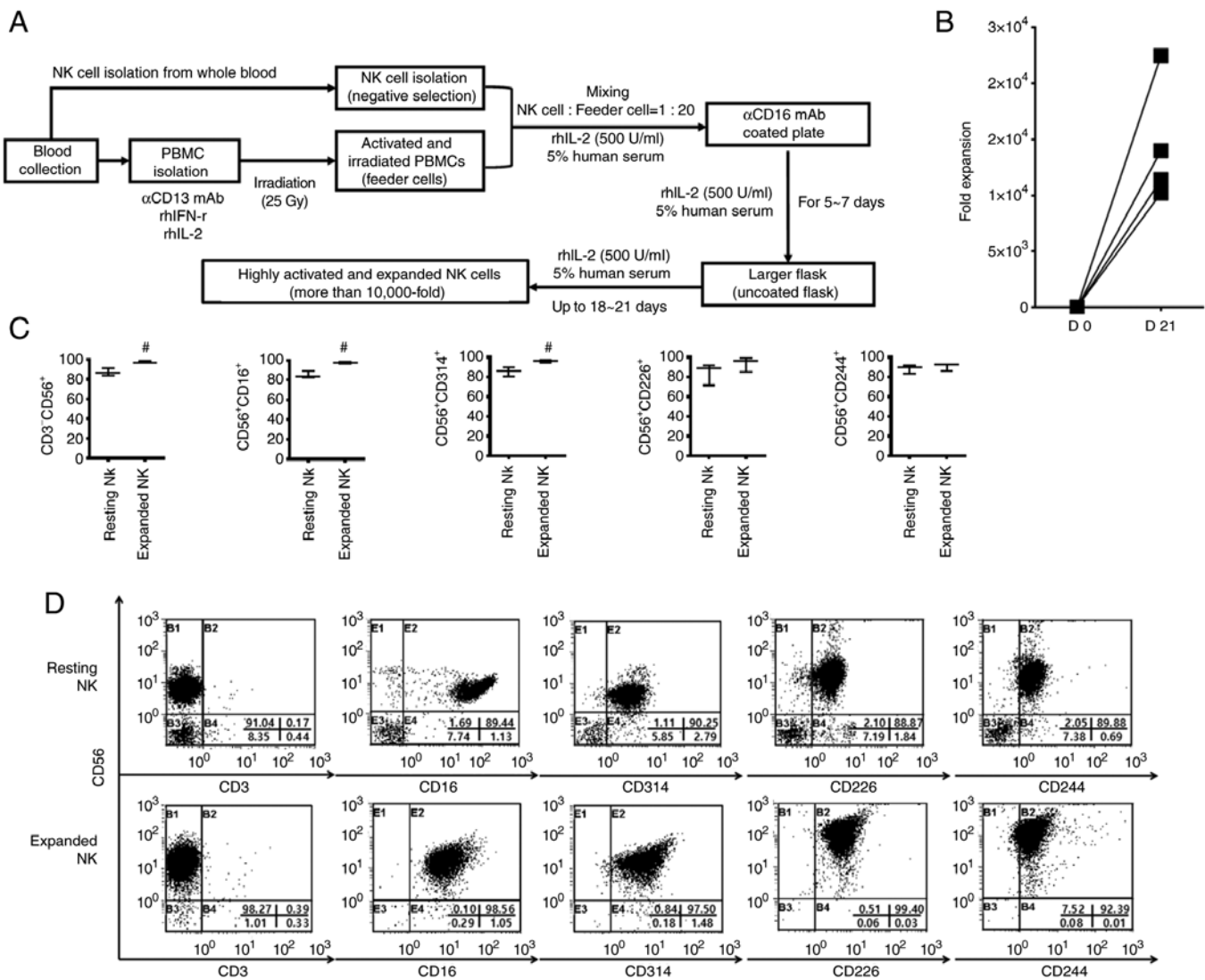


Figure 2. NK cell expansion is further enhanced through the stimulation of activated and irradiated PBMCs. NK cells isolated from healthy donors were expanded for 21 days using activated and irradiated PBMCs. (A) Schematic representation of large scale expansion of high purity activated NK cells by activated and irradiated PBMCs. (B) The expansion of NK cells was determined at 0 and 21 days. The results represent the mean \pm SD of 4 donors. NK cell surface antigens were analyzed by FCM. (C) Comparison of the expression levels of NK cell receptors between resting and expanded NK cells. Data are presented as the mean \pm SD of 3 donors. (D) Representative FCM dot plots of resting and expanded NK cells. Statistical significance was determined using paired Student's t-test. * $P < 0.05$ (resting NK cells vs. expanded NK cells). NK, natural killer; PBMCs, peripheral blood mononuclear cells; FCM, flow cytometry.

increased expression of MICA, MICB, ULBP1, ULBP2/5/6, CD48, and CD155 compared with those of untreated PBMCs. In particular, the expression levels of MICA, MICB, ULBP1, ULBP2/5/6, and CD155 on activated and irradiated PBMCs were significantly increased compared with those of PBMCs subjected to radiation alone. Therefore, radiation altered the expression levels of activating ligands in human PBMCs, and the combination of anti-CD3 mAb, IFN- γ , and IL-2 further promoted these alterations.

Activated and irradiated PBMCs potently induce the expansion of NK cells. To determine the expansion efficiency of NK cells by activated and irradiated PBMCs, NK cells isolated from the whole blood of healthy donors were expanded using activated and irradiated autologous PBMCs. As shown in Fig. 2, isolated NK cells expanded effectively *in vitro* during the culture period and expanded by >10,000-fold at 3 weeks

(Fig. 2A and B). In particular, T cell contamination, which can induce graft-vs.-host disease, was hardly observed in the expanded NK cells finally obtained (Fig. 2C and D). In addition, the expanded NK cells showed high expression of various activating receptors (Fig. 2C and D). These results indicated that the combination of anti-CD3 mAb, IFN- γ , and IL-2 with radiation potently promoted the expansion of NK cells by inducing the expression of various activating ligands of PBMCs.

Radiation increases the expression levels of various ligands associated with NK cell sensitivity in human colorectal cancer cells. The expression levels of various activating and inhibitory ligands were evaluated in SW480 and HT-29 human colorectal cancer cells following radiation. SW480 and HT-29 cells were harvested at 0, 24, or 48 h after irradiation at 4 or 8 Gy, and analyzed using FCM. The cell surface

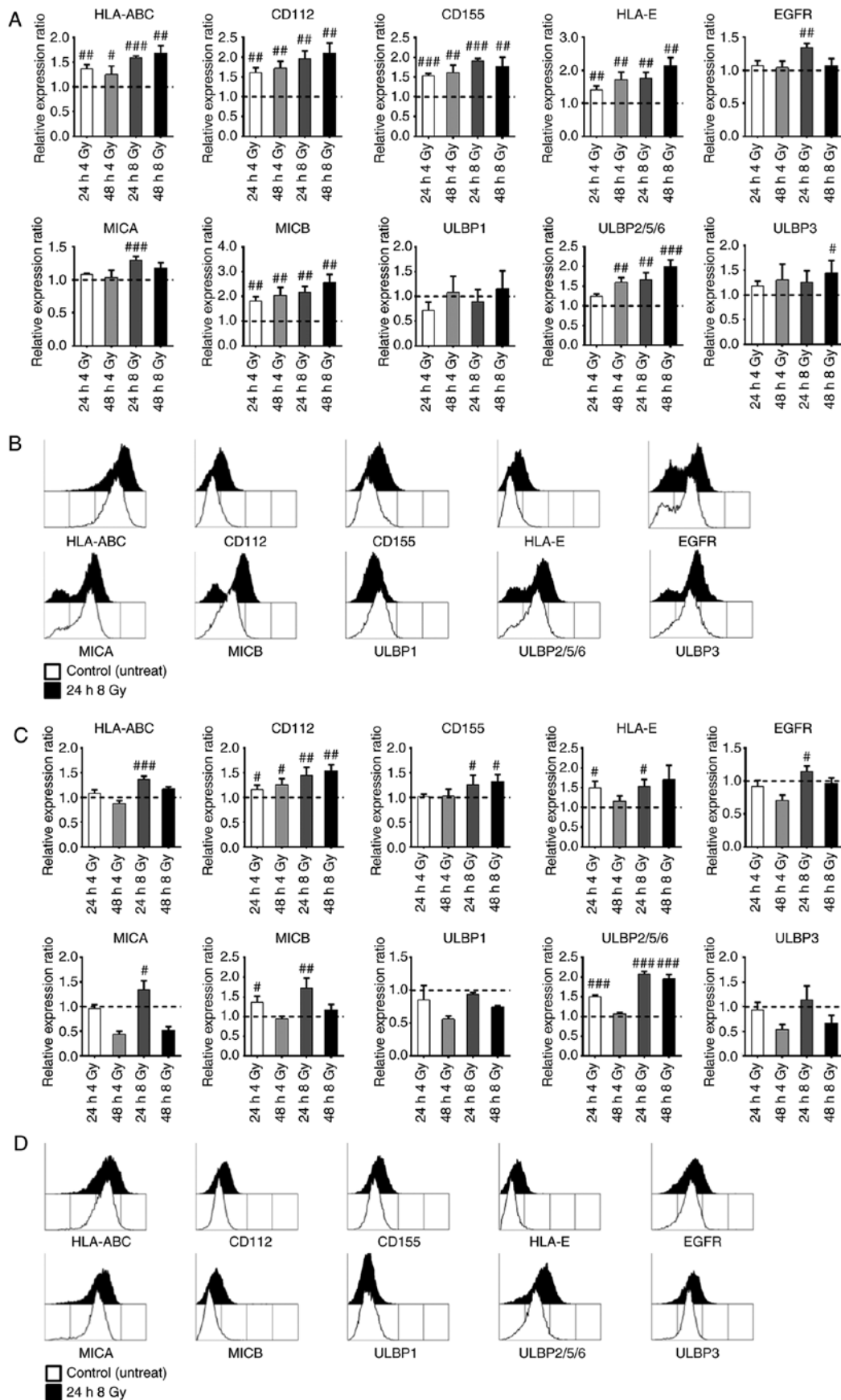


Figure 3. Radiation induces the expression of various natural killer-associated ligands in human colorectal cancer cells. SW480 and HT-29 cells were harvested at 0, 24, or 48 h after irradiation at 4 or 8 Gy. The expression of surface markers of cancer cells was analyzed by FCM. Ratios of MFIs obtained from irradiated SW480 and HT-29 cells and untreated controls. (A and C) Relative expression ratios were calculated by dividing the MFI of irradiated (A) SW480 or (C) HT-29 by that of the untreated control. (B and D) Representative FCM histograms (white, untreated cells; black, 24 h after 8 Gy irradiation) of (B) SW480 and (D) HT-29 cells. Data are presented as the mean \pm SD of 3 replicates. Statistical significance was determined using a paired Student's t-test. $^{\#}P<0.05$, $^{##}P<0.005$, $^{###}P<0.0005$ (untreated control vs. irradiated SW480 or HT-29 cells). MFI, median fluorescence intensity; FCM, flow cytometry.

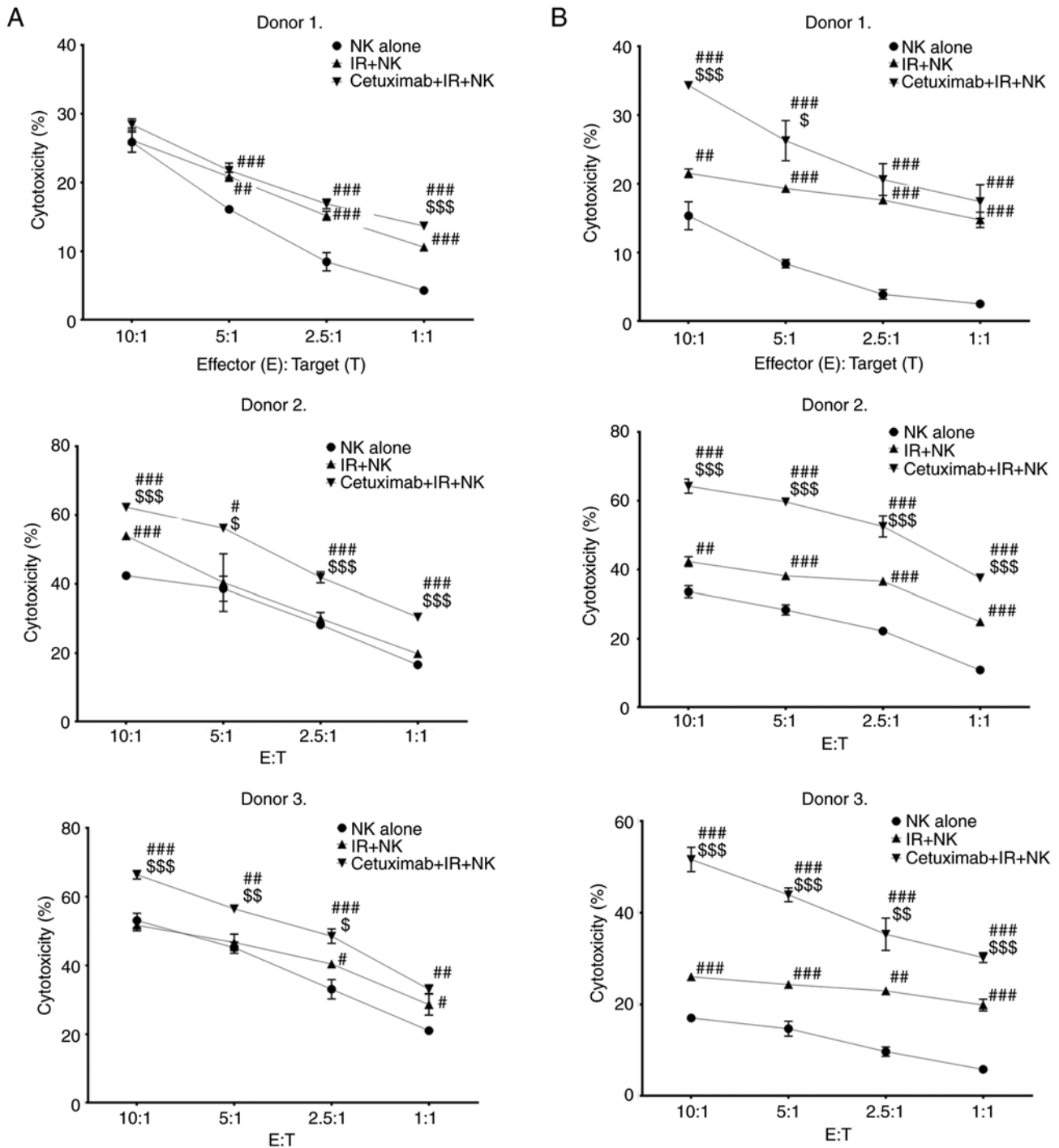


Figure 4. Combination treatment of radiation and cetuximab with expanded NK cells significantly improves cytotoxic activity against colorectal cancer cells. Cytotoxicity assays were performed using target (A) SW480 and (B) HT-29 cells. Data are representative of 3 healthy donors. Each experiment was performed in triplicate. Data are presented as the mean \pm SD of three repeats. Statistical significance was determined using a one-way ANOVA followed by a Tukey's post hoc test. # $P<0.05$, ## $P<0.005$, ### $P<0.0005$ (NK alone vs. other groups); \$ $P<0.05$, \$\$ $P<0.005$, \$\$\$ $P<0.0005$ (IR + NK vs. cetuximab + IR + NK). NK, natural killer; IR, ionizing radiation.

expression levels were quantified using MFIs. Relative expression ratios were calculated by dividing the 24 and 48 h samples' MFI by the untreated samples' MFI. As shown in Fig. 3, the HLA-ABC and HLA-E expression levels were increased by radiation in both SW480 (Fig. 3A and B) and HT-29 (Fig. 3C and D) cells. The EGFR expression level was increased only at 24 h after 8 Gy radiation, and then it decreased in both SW480 and HT-29 cells. The expression

levels of CD112, CD155, MICA, MICB, and ULBP2/5/6 were significantly increased by radiation in both SW480 and HT-29 cells. In particular, the expression levels of MICA, MICB, and ULBP2/5/6 showed a higher increase at 8 Gy of radiation in both SW480 and HT-29 cells, and there were more significant differences at 24 h after 8 Gy radiation in HT-29 cells. Therefore, these results indicated that radiation strongly increased the expression of various ligands that

modulate the sensitivity of NK cells in SW480 and HT-29 colorectal cancer cells.

Combination treatment using radiation and cetuximab along with expanded NK cells efficiently enhances cytotoxic activity against human colorectal cancer cells. The cytotoxic activity of expanded NK cells in the presence or absence of cetuximab and/or radiation was confirmed in SW480 and HT-29 human colorectal cancer cells. Expanded NK cells were co-cultured with the irradiated and/or cetuximab-coated human colorectal cancer cells at various ratios for 4 h. As shown in Fig. 4, despite the high expression of MHC class I of SW480 and HT-29 cells (Fig. 4), the expanded NK cells effectively lysed these colorectal cancer cells, and this antitumor cytotoxic activity was significantly enhanced by combination treatment of radiation. The combination of expanded NK cells and radiation showed higher cytotoxic activity compared with that of NK alone in HT-29 cells (Fig. 4B). However, the cytotoxic activity of the combination of expanded NK cells and radiation against SW480 (Fig. 4A) cells was relatively lower than that of HT-29 cells. This result may be due to the higher expression levels of MHC class I (HLA-A, -B, -C, and -E) molecules in SW480 cells by irradiation compared with that of HT-29 cells. Importantly, the combination treatment of radiation and cetuximab with expanded NK cells showed the strongest antitumor cytotoxic activity among all the treatments for SW480 and HT-29 cells. Taken together, these results indicated that expanded NK cells were capable of effectively removing human colorectal cancer cells, and the antitumor cytotoxic activity of expanded NK cells was further enhanced by cetuximab-mediated ADCC and radiation-induced activating ligands.

Discussion

Numerous studies have used irradiated cancer cells as feeder cells for NK cell expansion. However, since these methods may cause unforeseen complications due to the cancer cells used, their safety must be thoroughly verified before clinical application. Furthermore, the final product obtained by these methods is typically highly contaminated with unwanted T cells (21-25). In a previous study, our group reported a new method of large-scale expansion of potent NK cells (26). This method used an anti-CD16 mAb and irradiated autologous PBMCs without the use of cancer cell-derived feeder cells for the expansion of NK cells. In the present study, a novel method to more effectively expand NK cells was developed. PBMCs isolated from whole blood were activated by treatment with anti-CD3 mAb, IFN- γ , and IL-2, and then exposed to radiation. The activated and irradiated PBMCs further increased the expression levels of various activating ligands that increased the sensitivity of NK cells compared with PBMCs irradiated with radiation alone. A previous study reported that activation of T cells resulted in the expression of multiple NKG2D ligands (MICA, ULBP1, ULBP2, and ULBP3) through an Ataxia-telangiectasia mutated (ATM)/Ataxia-telangiectasia and Rad3-related (ATR)-dependent mechanism (30). Also, IFN- γ increased the expression of MIC molecules on monocytes (31). This method expanded high-purity activated NK cells by $\geq 10,000$ -fold with little contamination of T cells. The expanded NK cells exhibited upregulated expression of

various activating receptors and promoted the secretion of cytotoxic granules.

Colorectal cancer is the third leading cause of cancer and the second leading cause of cancer-associated mortality, and its annual incidence is gradually increasing worldwide (32). Surgery is the most effective treatment option for patients with early-stage colorectal cancer, but numerous patients are diagnosed in either metastatic or recurrent states, which are inoperable at the time of initial diagnosis. Recently, an EGFR-targeted antibody (cetuximab) was used to treat colorectal cancer in addition to conventional treatments, such as chemotherapy and radiotherapy; however, it is not recommended due to its low therapeutic effect when used alone (17,18). Therefore, our group developed a novel treatment approach using a combination of expanded NK cells with radiotherapy to overcome the shortcomings of this targeted antibody therapy and enhance its effects.

The present study investigated the combined effect of cetuximab and radiation with expanded NK cells using SW480 and HT-29 human colorectal cancer cells. These colorectal cancer cells are not only highly resistant to cetuximab monotherapy but also resistant to NK cells due to their higher HLA-E expression levels compared with those of other human colorectal cancer cells such as COLO320, Caco-2, and SW620 (29,33). A synergistic antitumor effect was observed when expanded NK cells were used in combination with cetuximab and radiation.

Radiation increased the expression levels of various ligands that modulate NK cell sensitivity in SW480 and HT-29 colorectal cancer cells. In particular, the expression levels of activating ligands such as DNAM-1 and NKG2D ligands were further increased by radiation. Also, the current study investigated the effect of radiation on EGFR expression in SW480 and HT-29 cells; however, further studies are needed to fully understand the effect of radiation on EGFR expression. The cytotoxic activity of the expanded NK cells may have been synergistically enhanced, as radiation increases the sensitivity of NK cells to cancer cells, and EGFR-targeted antibody (cetuximab) induces ADCC. Irradiation of cancer cells resulted in a variety of biological changes that increased the responsiveness of NK cells (6). In particular, radiation played an important role in recruiting NK cells to the tumor site, thus providing various activation signals (6,34). Importantly, the DNA damage response by radiation increased the expression of DNAM-1 and NKG2D ligands, which stimulated the sensitivity of NK cells to cancer cells by activating the ATM/ATR signaling pathway, leading to enhanced cancer cell killing effect (9,35,36).

ADCC is a key factor in the clinical efficacy of therapeutic antibodies (12). A significant correlation between ADCC and clinical response to treatment with therapeutic antibodies in various patients with cancer was previously reported (27-29). Furthermore, it was demonstrated that antibody-resistant cancer cells were effectively eliminated by ADCC. The susceptibility to ADCC was similar in both antibody-sensitive and antibody-resistant cells (37). Therefore, the administration of expanded NK cells may further enhance the clinical efficacy of therapeutic antibodies by inducing potent ADCC.

NK cells recognize their targets independent of human leukocyte antigen, which greatly reduces the risk of

graft-vs.-host disease (38). These characteristics of NK cells provide unique advantages for allogeneic therapeutic applications (39). Previous studies have shown that the adoptive transfer of NK cells following *ex vivo* activation and expansion is safe and well-tolerated in various cancer patients (39-43), with fever and fatigue being the most commonly reported side effects. Therefore, similar side effects are expected in colorectal cancer patients receiving adoptive NK cell transfer.

Soft tissue sarcoma (STS) is correlated with the expression of programmed cell death-1 (PD-1), PD ligand-1 (PD-L1), New York esophageal squamous cell carcinoma-1 (NY-ESO-1), and melanoma-associated antigen-A4 (MAGE-A4), which may indicate a poor prognosis (44). Activated NK cells express PD-1, and PD-1/PD-L1 blockade can increase NK cell production of IFN- γ and CD107a, as well as the anti-tumor effect of NK cells (45). Combining NK cells with immune checkpoint inhibitors (anti-PD-1 or anti-PD-L1 mAb) may effectively inhibit STS. While NK cells do not directly recognize NY-ESO-1 and MAGE-A4 antigens like T cells, they can still inhibit STS by binding to ligands expressed in the tumors (46). Combining specific antibodies that bind to NY-ESO-1 or MAGE-A4 with NK cells may have ADCC and direct effects on NK cells.

One potential limitation to consider is the lack of a normal cell line for comparison. Without a normal cell line, it may be challenging to determine the extent to which the observed effects are specific to the experimental conditions used. Another limitation to consider is the lack of *in vivo* validating experimental data. While *in vitro* studies can provide valuable insights into cellular processes, they may not fully reflect the complexity of physiological systems *in vivo*. However, in this study, the efficacy of *in vitro* activated and expanded NK cells was confirmed using two types of colorectal cancer cell lines. In future studies, the effectiveness of *in vitro* activated and expanded NK cells will be verified using a variety of colorectal cancer cell lines, and their efficacy and safety *in vivo* will also be determined using severe combined immunodeficiency disease (SCID) mice. Through these efforts, we aim to provide direction for future research and address the limitations of *in vitro* studies.

In summary, a novel method to efficiently expand high-purity NK cells with a potent antitumor activity using activated and irradiated autologous PBMCs was developed in the present study. Combination treatment of radiotherapy and cetuximab together with the expanded NK cells was demonstrated to be an effective method to inhibit human colorectal cancer cells. Therefore, this multimodal approach may help to design strategies for eradicating cancer cells in the clinical setting of NK cell-based immunotherapy. A combination of conventional treatments and/or targeted anticancer agents with NK cell-based immunotherapy could be a promising strategy to enhance the immune response and overcome the limitations of current treatments for colorectal cancer. This approach could increase survival rates and improve the quality of life of patients, particularly in patients with advanced-stage cancer.

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Availability of data and materials

Raw data were generated at Dongnam Institute of Radiological & Medical Sciences. Derived data supporting the findings of the present study are available from the corresponding author on reasonable request.

Authors' contributions

YSP and JHB designed the study. EKK, HRL and WCS designed the experimental methods, performed the experiments and wrote the manuscript. GYP performed the data analysis. WCS and YSP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Experiments using human blood were approved (approval no. D-2002-032-002) by the IRB of Dongnam Institute of Radiological & Medical Sciences (Jangan-eup, South Korea), and written informed consent was obtained from all the donors before involvement in the study.

Patient consent for publication

Written informed consent was obtained from the donors for publication of the data.

Competing interests

The authors declare that they have no competing interests.

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