Combined Treatment With Dendritic Cells and 5-fluorouracil Elicits Augmented NK Cell-mediated Antitumor Activity Through the Tumor Necrosis Factor-α Pathway

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Summary: Antitumor effects and mechanism of combined therapy with a dendritic cell (DC) vaccine and fluorouracil (5-FU) were investigated. Cytotoxic activity against MC38 cells, untreated or pretreated with 5-FU, was examined in splenocytes from mice inoculated with DCs: DCs pulsed with MC38 lysate or treated with LPS or both and untreated DCs. Inoculation with all types of DCs induced the significant cytotoxic activity of splenocytes, and pretreatment of MC38 cells with 5-FU significantly enhanced the cytotoxic activity of splenocytes. Depletion of natural killer (NK) cells, but not of $CD8^+$ or $CD4^+$ T cells, in the splenocytes from DC (without MC38 lysate-pulse or LPS treatment thereafter)-inoculated mice decreased the cytotoxic activity. The cytotoxic effect was eliminated by treatment with a monoclonal antibody (mAb) against tumor necrosis factor (TNF)-α and was partially inhibited by concanamycin A. Inoculation of mice with DCs upregulated TNFα expression on NK cells. MC38 cells pretreated with 5-FU exhibited enhanced expression of procaspase 8 and efficiently underwent apoptosis by TNF-α with activation of caspase 8. Although treatment with 5-FU upregulated Rae-1 expression on MC38 cells, the NK-cell–mediated cytotoxic activity was not suppressed by treatment with an anti–Rae-1 mAb or an antinatural killer group 2D mAb or both. These results indicate that combined therapy with a DC vaccine and 5-FU is a promising strategy for cancer treatment mediated by the tumoricidal activity of NK cells through the TNF-α pathway.

Key Words: dendritic cell, natural killer cell, tumor necrosis factor-α, 5-fluorouracil, caspase-8

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Dendritic cells (DCs) are potent antigen-presenting cells that play a pivotal role in the development of adaptive immunity by priming naive T cells and inducing antigen-specific T-cell responses.[1,2] Although DCs can induce antitumor immunity mediated by tumor-antigen-specific CTLs,[3,4] results of treatment with many cancer immunotherapies using a DC vaccine alone have been unsatisfactory.[5,6] Accordingly, novel strategies are needed to make DC-based cancer immunotherapy more effective.

Some chemotherapeutic agents might enhance antitumor immune activity, and, conversely, immunotherapy might decrease the resistance to chemotherapy of tumor-bearing hosts.[7] The chemotherapeutic agents fluorouracil (5-FU), irinotecan, cisplatin, and dacarbazine sensitize tumor cells to CTLs.[8,9] Cisplatin and several proteasome inhibitors sensitize tumor cells to natural killer (NK) cells.[10–12] Chemotherapeutic drugs might activate apoptosis-inducing pathways mediated by death-receptor signal transduction such as Fas/Fas ligand (Fas L) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor interaction or might increase the sensitivity to perforin/granzyme activity.[8,11] Combination therapy with death-receptor ligands, such as Fas L, TRAIL, and TNF-α, and chemotherapeutic agents, such as cisplatin, 5-FU, topotecan, doxorubicin, paclitaxel, and vincristine, show synergistic effects against malignant tumors, and activation of caspase 8 by chemotherapeutic agents might be closely associated to the synergistic effects by death-receptor ligands and chemotherapy.[13,14]

The fluoropyrimidine 5-FU has been a key drug in the treatment of colorectal cancer. Standard treatments for advanced colorectal cancer have long been based on 5-FU in combination with leucovorin,[15] and 5-FU/leucovorin plus oxaliplatin or irinotecan are new standard regimens.[16] Oral fluoropyrimidines, such as uracil/tegafur, capecitabine, and S-1, have similar effects and tolerability to intravenous 5-FU/leucovorin treatment.[17,18] An effective combination therapy with 5-FU and immunotherapy would be extremely valuable for the treatment of colorectal cancer.

In this study, we examined the antitumor activity mediated by NK cells and induced by combination treatment with DCs and 5-FU against MC 38 colorectal cancer cells. We analyzed the mechanism of the tumoricidal activity of this combined treatment.

MATERIALS AND METHODS

Mice and Cell Lines

Eight-week-old female C57BL/6 (B6) mice were supplied by Nihon SLC Co., Ltd. (Tokyo, Japan). All

animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science. Cells of the MC38 line (murine colorectal cancer) were gifted from Prof. Donald Kufe in 1998, and maintained as monolayer cultures in Dulbecco modified Eagle medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated fetal bovine serum.

Agents

Recombinant murine (rm) granulocyte/macrophage colony-stimulating factor (GM-CSF), rm interleukin (IL)-4 and recombinant human (rh) TNF-α were purchased from Peprotech EC Ltd (London, UK). A FasL-neutralizing monoclonal antibody (mAb), a TRAIL-neutralizing mAb (N2B2), and a TNF-α–neutralizing mAb (MP6-XT22) were prepared as described earlier.[19,20] A phycoerythrin(PE)-labeled antimouse TNFR type I mAb was purchased from BioLegend (San Diego, CA). A fluorescein isothiocyanate (FITC)-labeled anti-DX5 (NK cells) mAb was purchased from Pharmingen (San Diego, CA). A rabbit anticaspase 8 p18 polyclonal antibody (pAb) (H-134) and a rabbit anticaspase 9 pAb (1-134) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Calbiochem (Darmstadt, Germany), respectively. Antimouse Rae-1 mAB (clone 199205) and anti-Naturak killer group 2D (NKG2D) mAb (clone 191004) were purchased from R&D Systems, Inc. (Minneapolis, MN).

Preparation of DCs

DCs were prepared according to the method of Inaba et al[21,22] with certain modifications. In brief, after overnight incubation of bone marrow cells, nonadherent cells were removed by washing and adherent cells were incubated in a medium containing 10 ng/mL of rm GM-CSF and 10 ng/mL of rmIL-4 for 6 days. The resulting cells were a DC-rich fraction in which 70% to 80% of cells showed DC features (Expression of CD11c, CD80, CD86, and MHC class II).

Treatment of DCs and Inoculation of DCs into Mice

The DCs were unpulsed or were pulsed with MC38 lysate, generated by freezing and thawing the MC38 cells 3 times, for 24 hours (DC:MC38, 1:1). The DCs were untreated or were treated with lipopolysacharide (LPS, 1 μg/mL) for an additional 24 hours. The DCs were injected subcutaneously into the backs of female C57BL/6 mice (5×10^5 cells per mouse) twice with a 1-week interval.

Cytotoxic Assay

Spleens were obtained from DC-treated mice or from untreated mice 1 week after the second injection. Splenocytes were obtained as described earlier.[23] Splenocytes from the mice were cultured for 3 days in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L of glutamate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The resulting splenocytes were collected and passed through nylon wool columns to obtain effector cells. In some experiments, purified NK cells were used as effector cells. The cytotoxic activity of effector cells was determined with an Active-caspase-3 Apoptosis kit I (Pharmingen BD Biosciences, San Diego, CA)[24,25] according to the manufacturer's instruction or with a ^{51}Cr-release assay, as described earlier.[23] Both assay methods have shown the same cytotoxic activity of immune cells against MC38 cells. Cytotoxic activity was determined according to the formula of (Experimental release-Spontaneous release/Maximum release-Spontaneous release) × 100. As toxicity of chemotherapeutic agents themselves was represented as spontaneous release of ^{51}Cr from the agent-treated target cells, the results represent the cytotoxic activity mediated by cytocydal activity of effecter cells.

Target MC38 cells were untreated or treated with various chemotherapeutic agents before the cytotoxic assay. By using MTT assay, concentration of each agent to inhibit the cell proliferation to 50% of untreated control in 48 hours was determined for pretreatment of target cells.

Depletion and Purification of Immune Cells

To obtain cell fractions depleted of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells, a magnetic cell isolation kit (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) was used according to the manufacturer's instructions. About 90% of $CD4^+$ and $CD8^+$ T cells were eliminated from the splenocytes by this method. To isolate purified NK cells, a magnetic NK cell isolation kit (Miltenyi Biotec) was used according to the manufacturer's instructions. The purity of the isolated NK cells was greater than 90%.

Real-time Reverse-transcription Polymerase Chain Reaction (PCR) for Expression of the TNF Receptor

Real-time reverse-transcription PCR was done with the 7300 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). RNA was extracted from MC38 cells with the acid guanidinium thiocyanate-phenol-chloroform extraction method (Isogen, Nippon Gene, Tokyo, Japan). Single-stranded cDNA was synthesized from 2 μg of total RNA with the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) in 20 μL of reaction mixture at 37°C for 2 hours. Gene expression was analyzed with Power SYBR Green PCR master mix (Applied Biosystems). The PCR conditions consisted of an initial denaturation step at 95°C for 10 seconds, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. The sequences of primers for TNFR type I and glyceraldehyde-3-phosphate dehydrogenase were as: TNFR type I, 5′-TTCAACGG CACCGTGACAA-3′ and 5′-AACCCTGCATGGCAGTT ACACA-3′; and glyceraldehyde-3-phosphate dehydrogenase, 5′-ATGGTGAAGGTCGGTGTGAA-3′ and 5′-AAT GAAGGGGTCGTTGATGG-3′.

TNFR-mediated Apoptosis In Vitro

MC38 cells were fixed in 70% precooled ethanol, and incubated in PBS containing 10 μg/mL propidium iodide and 10 μg/mL RNase A for 20 minutes at room temperature. Fluorescence intensity was measured with a FACS-Calibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA).

Western Blot Analysis

Protein samples were separated with 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking, the membranes were incubated with antibodies against either caspase 8 or caspase 9. Each membrane was then probed for 120 minutes with peroxidase-conjugated anti-rabbit Ig (Amersham Biosciences, UK,

Buckinghamshire, UK) and analyzed with an enhanced chemiluminescence detection system (Amersham Biosciences, UK).

Statistical Analysis

The significance of difference among the groups was analyzed with Student *t* test for 2 independent groups and with Tukey test for multiple group comparisons.

RESULTS

Cytotoxic Activity of Splenocytes From DC-inoculated Mice Was Enhanced by Pretreatment of Target MC 38 Cells With Chemotherapeutic Agents

Inoculation of mice with each type of treated DC induced substantial cytotoxic activity although inoculation of tumor lysate pulsed DC induced significantly higher cytotoxic activity than the others (Fig. 1A). This result indicates that inoculation with DCs without loading tumor antigen could induce the cytotoxic activity. Pretreatment of MC38 cells with 5-FU significantly enhanced the cytotoxic effect of splenocytes from mice treated with each type of DC (Fig. 1A), and the cytototic activity was effecter: target cell ration dependent (Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A49). As we focused on the cytotoxic activity induced by DCs irrelevant of tumor antigen-specific T-cell response, we used DCs without tumor antigen pulse or LPS treatment for inoculation into mice thereafter and such mice are shown as "DC-treated mice."

As with pretreatment with 5-FU, pretreatment of MC38 with mitomycin C (MMC), or irinotecan (CPT-11) significantly enhanced the cytotoxic effect of splenocytes from DC-treated mice, whereas treatment with cisplatin (CDDP) did not enhance the cytotoxic effect (Fig. 1B).

As 5-FU is the only 1 of these agents that can be administered orally and can maintain stable serum

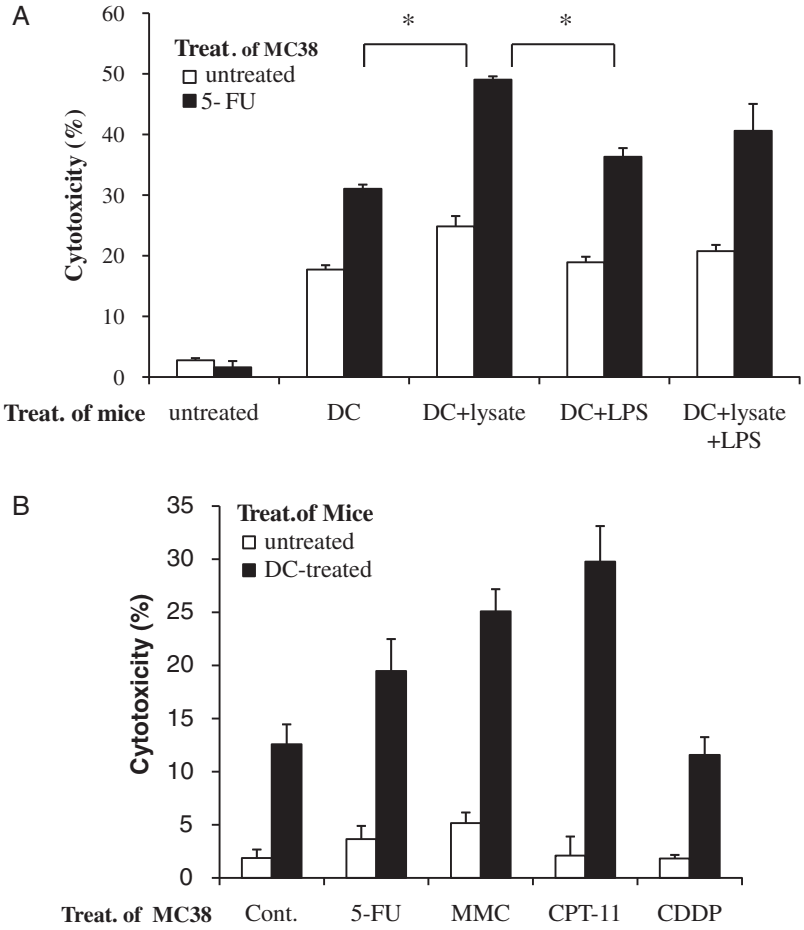

FIGURE 1. A, Inoculation of DCs into mice induced cytotoxic activity of splenocytes against MC38 cells and pretreatment of MC38 cells with 5-FU enhanced the splenocyte-mediated cytotoxic activity. Bone-marrow–derived DCs were untreated or were pulsed with MC38 lysate or treated with lipopolysacharide (LPS) or both. Treated DCs were injected subcutaneously into mice on days 1 and 7, and splenocytes were collected on day 14. These splenocytes were examined for cytotoxic activity against MC38 cells, either untreated or pretreated with 5-FU (2.3 μM for 48 h) (effector:target ratio = 40:1). All experiments were done in triplicate and repeated at least 3 times. *$P < 0.01$. B, Treatment with not only 5-FU but mitomycin C (MMC) or CPT-11 augmented the cytotoxic activity of splenocytes from DC-inoculated mice against MC38 cells. Splenocytes obtained from untreated mice and from mice treated with DCs (without tumor lysate pulse or LPS treatment) were examined for cytotoxic activity against MC38 cells (effector:target ratio = 40:1). MC38 cells were pretreated with 5-fluorouracil (FU), MMC (1.4 μM for 48 h), CPT-11 (140 nM for 48 h), or CDDP (1 μM for 48 h).

concentrations as compared with other agents administered intravenously, we chose 5-FU to be combined with immunotherapy.

Cytotoxic Activity Against MC38 Cells Induced by DC-inoculation Was Mediated by NK Cells

Cytotoxic activity was not suppressed by depletion of $CD4^+$ or $CD8^+$ T cells from splenocytes (Fig. 2A-a) but was significantly decreased by depletion of NK cells from the splenocytes (Fig. 2A-b); this finding indicates that NK cells are involved in the cytotoxic activity. Furthermore, splenocytes from DC-treated mice showed high cytotoxic activity against NK-cell–sensitive Yac-1 target cells (Supplementary Fig. 2, Supplemental Digital Content 2, http://links.lww.com/JIT/A50).

The NK cells from mice treated with DCs elicited higher cytotoxic activity against MC38 cells than did NK cells from untreated mice (Fig. 2B-a). Furthermore, NK cells from DC-treated mice showed greater cytotoxic activity against 5-FU–pretreated MC38 cells than against untreated MC-38 cells (Fig. 2B-b).

Augmentation of Cytotoxic Activity by 5-FU Is Mediated by TNF-α

Treatment with mAbs against TNF-α and Concanamycin A (CMA) significantly inhibited cytotoxic activity, and the inhibitory effects of mAbs against FasL and TRAIL were not significant (Fig. 3). These results indicate that augmentation of NK-cell–mediated cytotoxic activity

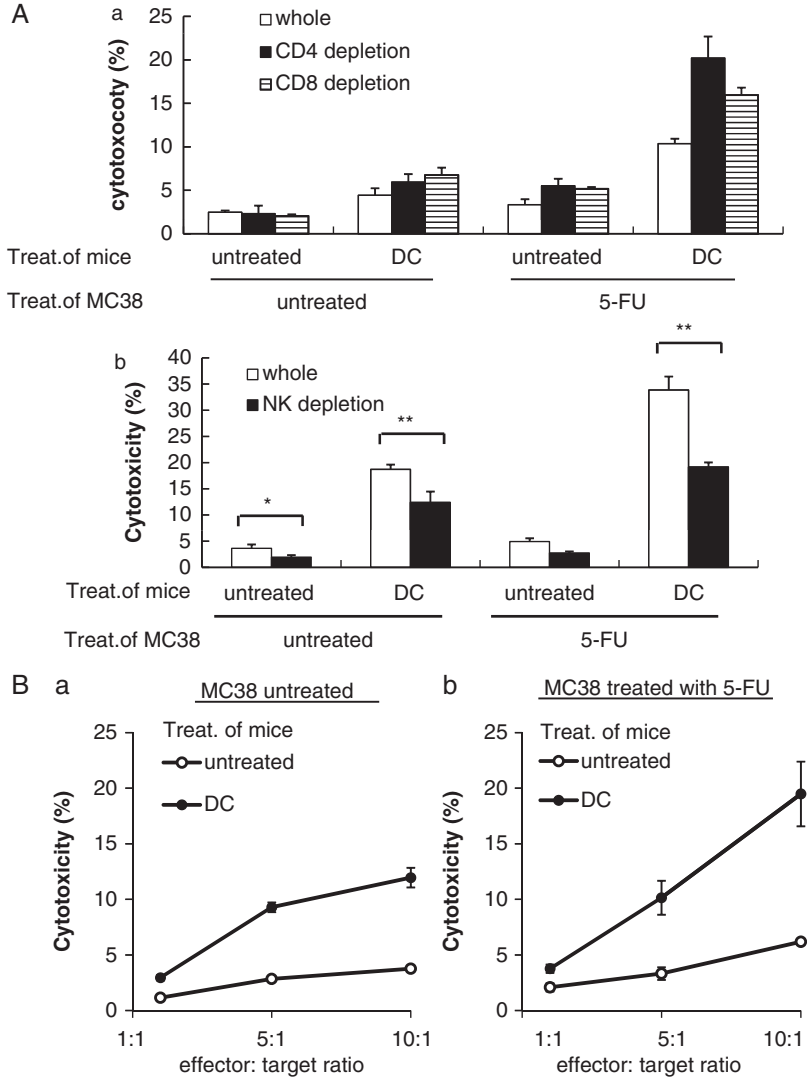

FIGURE 2. A, Cytotoxic activity eliminated by depletion of natural killer (NK) cells. Splenocytes were collected from untreated mice or mice treated with dendritic cells (DCs) without tumor lysate pulse or LPS treatment. $CD4^+$ T cells, CD8+ T cells (a), and NK cells (b) were depleted from splenocytes with a magnetic sorting system. Resultant splenocytes were examined for cytotoxic activity against MC38 cells pretreated with 5-FU (effector:target ratio = 40:1). All experiments were done in triplicate. *not significant, $^{**}P < 0.001$. B, Inoculation with DCs enhanced the cytotoxic activity of NK cells against MC38 cells, and treatment of target MC38 cells with 5-FU enhanced NK-cell–mediated cytotoxic activity. Splenocytes were collected from untreated mice or mice treated with DCs. NK cells were isolated from splenocytes with a magnetic sorting system. The cytotoxic activity of purified NK cells against untreated MC38 cells (a) or MC38 cells pretreated with 5-FU (b) were examined at the indicated effector:target ratio.

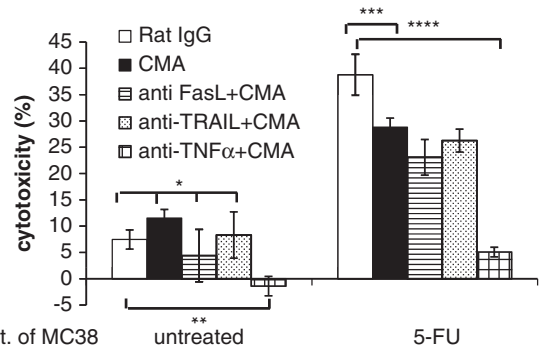

FIGURE 3. Augmentation of cytotoxic activity by 5-FU is mediated by TNF-α. NK cells from mice treated with Dcs were examined for cytotoxic activity against untreated MC38 cells or 5-FU-pretreated MC38 cells in the presence or absence of an anti-TNF-α mAb (10 μg/mL) and Concanamycin A (CMA) (100 nM), an anti-FasL mAb (10 μg/mL) and CMA, an anti-(TNF)–related apoptosis-inducing ligand (TRAIL) mAb (10 μg/mL) and CMA, or CMA alone (effector:target ratio = 10:1). All experiments were carried out in triplicate and repeated at least 3 times. *not significant, $^{**}P < 0.05$, $^{***}P < 0.01$, $^{****}P < 0.001$.

by 5-FU is mediated mainly by TNF-α interaction and partially by perforin/granzyme interaction.

DC Inoculation Enhanced the Expression of TNF-α on NK Cells

DC inoculation to mice enhanced the expression of TNF-α on NK cells in splenocytes (Fig. 4).

Five-FU Sensitizes MC38 Cells to TNF-α

The sub–G1 apoptotic population was significantly larger in MC 38 cells treated with both 5-FU and TNF-α than in MC 38 cells that had been untreated or had been treated with 5-FU or TNF-α alone (Fig. 5A).

No marked changes in TNFR expression were seen in MC38 cells treated with 5-FU (Fig. 5B-a). Expression of mRNA encoding TNFR type I in MC38 cells did not change with 5-FU treatment (Fig. 5B-b).

Caspase 8 was activated in MC38 cells by combination treatment with 5-FU and TNF-α but not by treatment with either agent alone (Fig. 5C). The expression of procaspase 8 was increased in MC38 cells treated with 5-FU, but active caspase 9 expression was not induced by 5-FU or TNF-α.

Augmentation of NK Cell-mediated Cytotoxic Activity by 5 FU Was not Associated With the Expression of MHC Class I on MC38 Cells or NKG2D/NKG2D-ligand Interaction

NK cells can kill target cells expressing few or no MHC class I molecules, which function as killer-inhibitory receptors for NK cells.[26,27] No marked changes in the expression of H-2K^b were seen after treatment of MC38 cells with 5-FU (Supplementary Fig. 3, Supplemental Digital Content 3, http://links.lww.com/JIT/A51).

NKG2D is an NK-cell–activating receptor expressed on NK cells, and NKG2D-ligand expression on tumor cells is enhanced by treatment with cytotoxic drugs.[27] The expression of Rae-1, a NKG2D ligand on MC38 cells, was enhanced by treatment of MC 38 cells with 5-FU (Fig. 6A). However, cytotoxic activity was not inhibited when an anti–Rae-1 neutralizing mAb (199205) or an anti-NKG2D neutralizing mAb (191004) or both were added to the cytotoxic assay (Fig. 6B).

DISCUSSION

Antitumor activity induced by a tumor-antigen-loaded DC vaccine is chiefly mediated by antigen-specific CTLs. However, we found that subcutaneous inoculation with DCs, not pulsed with tumor lysate or treated with LPS, activated NK-cell–mediated antitumor immunity against MC38 cells. NK cells are an important type of immune cell supporting the innate immune system and are thought to exhibit nonspecific cytotoxic activity without a specific stimulus for NK cell activation.[28] However, DCs have recently been reported to activate NK cells and trigger antitumor immunity mediated by NK cells.[29] The DC-dependent NK-cell activation is induced by DC-NK cell contact[30–32] or by cytokines secreted from DCs or by both.[32–38] The interaction between NKG2D-ligand–NKG2D and Jagged2-Notch provided by DC-to-NK cell contact can lead to NK-cell activation.[37,39,40] Although the ability

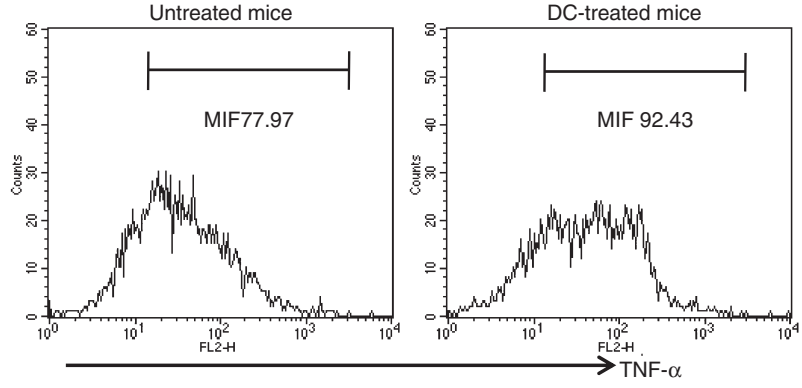

FIGURE 4. NK cells from DC-inoculated mice showed enhanced TNF-α expression. Splenocytes were collected from untreated mice or DC-inoculated mice, cultured for 3 days, and passed through nylon wool columns. The cells were stained with a fluorescein isothiocyanate-conjugated anti-DX5 mAb and a rat anti–TNF-α mAb, followed by a biotinylated goat anti-rat IgG Ab and phycoerythrin (PE)-conjugated streptavidin. Expression of TNF-α on the gated DX5$^+$ cells was examined with flow cytometry. Bars in the upper portion of the figure boxes show the area of positive staining. MIF: mean intensity of fluorescence.

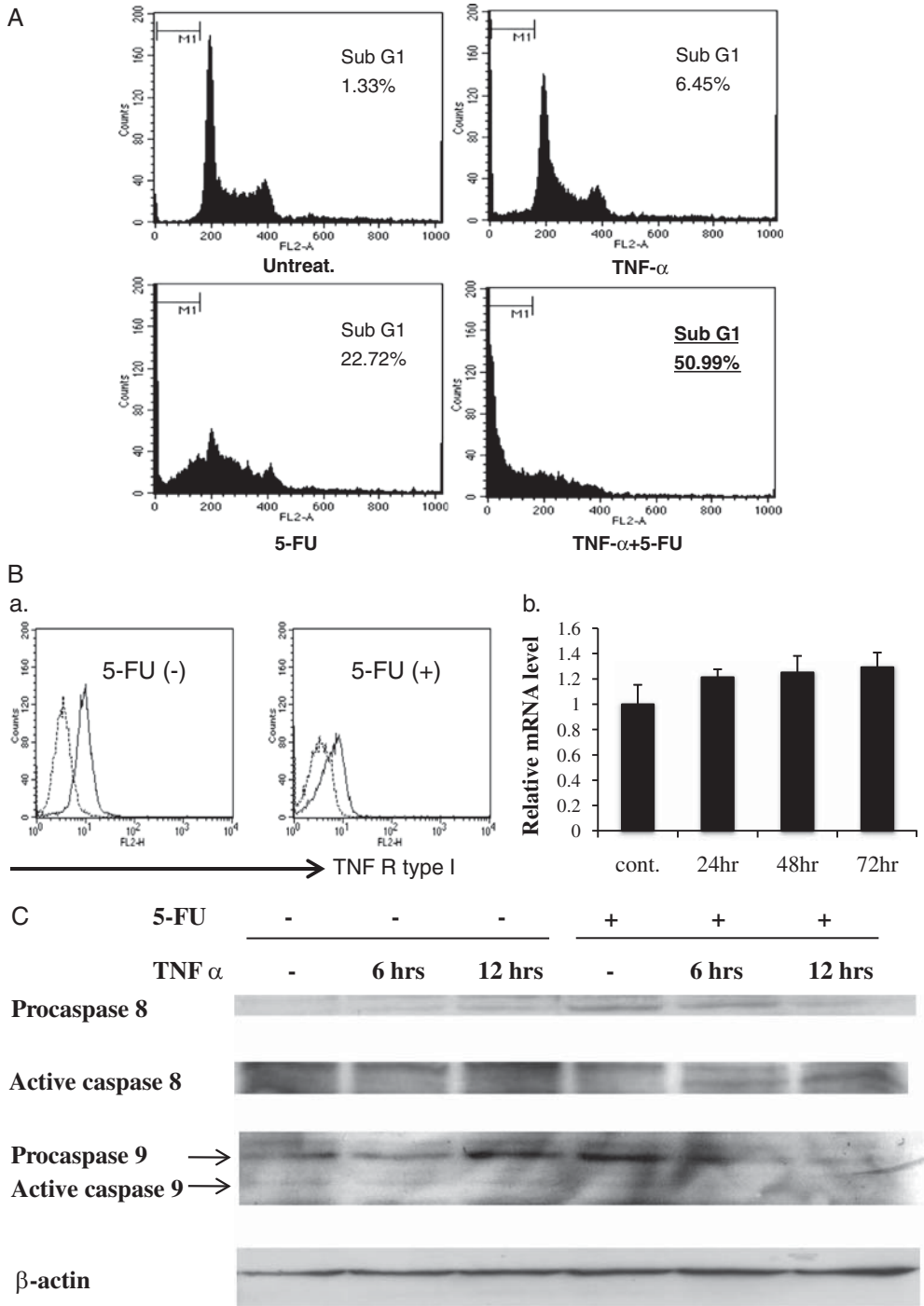

FIGURE 5. 5-FU sensitizes MC38 cells to TNF-α. A, Treatment with 5-FU and TNF-α caused cells to accumulate in the sub–G1 phase. The cell-cycle status of MC38 cells treated with 5-FU (2.3 μM) for 48 hours and then with rhTNF-α (50 ng/mL) (13) for an additional 24 hours was examined with flow cytometric analysis. Percentages indicate the sub–G_1 populations for each treated group. B, No marked changes in TNFR expression in MC38 cells treated with 5-FU. (a) Expression of TNF receptor (TNFR) type I in MC38 cells treated with 5-FU (2.3 μM) for 48 hours determined with flow cytometry. Dotted line, isotype-matched control; solid line TNFR type I. (b) Expression of TNFR type I mRNA in MC38 cells treated with 5-FU (2.3 μM) for indicated periods determined with quantitative real-time PCR. C, 5-FU upregulates expression of procaspase 8, and combined treatment with 5-FU and TNF-α activates caspase 8. MC38 cells were exposed to 5-FU (2.3 μM) for 48 hours and then to rhTNF-α (50 ng/mL) for an additional 6 to 12 hours. Changes in the expression and processing of caspases 8 and 9 were analyzed with Western blotting.

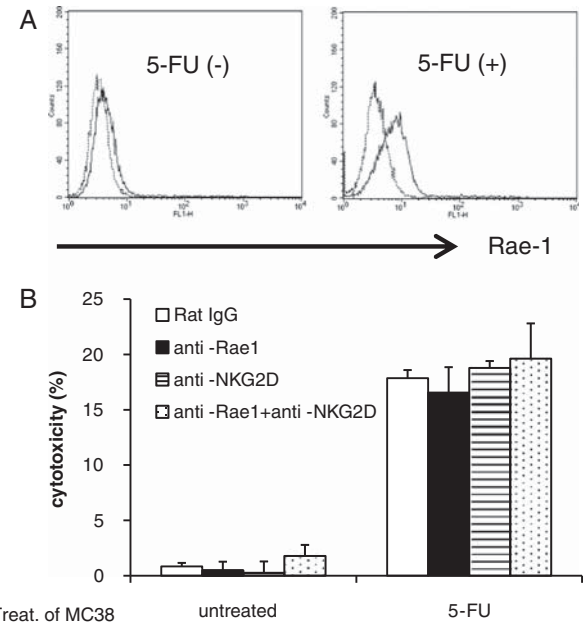

FIGURE 6. Treatment with 5-FU upregulated Rae-1 expression, but natural killer group 2D (NGK2D)/NKG2D-ligand interaction was not involved in NK-cell–mediated cytotoxic activity. A, MC38 cells were treated with 5-FU (2.3 μM for 48 h) and examined for Rae-1 expression with flow cytometry. B, The NK-cell-mediated cytotoxic activity against MC38 cells was examined in the presence of an anti–Rae-1 mAb or an anti-NKG2D mAb or both or in their absence. All experiments were carried out in triplicate and repeated at least 3 times.

to activate NK cells differs with the maturation status of DCs,[41,42] sufficient NK-cell activation was achieved by inoculation with immature DCs in this study.

The augmented tumoricidal activity generated by NK cells activated by DC inoculation and treatment of target MC38 cells with 5-FU was mediated mainly by TNF-α interaction, because the activity was significantly inhibited by treatment with an anti–TNF-α mAb but not with an anti–Fas L or anti–TRAIL mAb. Perforin/granzyme was involved in this augmented tumoricidal activity. As TNFR expression was not changed by treatment with 5-FU, a change in signal transduction through TNFR might be associated with this augmented tumoricidal activity. Furthermore, apoptosis of 5-FU–pretreated MC38 cells was efficiently induced with activation of caspase 8 when they were treated with rhTNF-α in vitro. This phenomenon might be closely associated with enhanced expression of procaspase 8 induced by 5-FU treatment. Caspase 8 expression in tumor cells is upregulated by treatment with chemotherapeutic agents through the P53 response,[14] and the synergistic effect of chemotherapy and death-receptor ligand occurs through activation of caspase 8.[30]

The cytocidal activity of NK cells is regulated by signal transduction through inhibitory receptors and activating receptors. The MHC class I molecule expressed on target cells is a ligand of NK cell inhibitory receptors and suppresses the cytocidal activity of NK cells.[26,27] In this study, expression of MHC class I molecules on MC38 cells was not affected by treatment with 5-FU. Furthermore, Rae-1 and MHC class I chain-related A are ligands of NKG2D, an NK-cell–activating receptor, and expression of these molecules on target cells enhances the cytocidal activity of NK cells.[27] In this study, expression of Rae-1 on MC 38 cells was significantly enhanced by treatment with 5-FU. Expression of the NKG2D ligands Rae-1 and MHC class I chain-related A are reportedly upregulated by DNA damage.[43] As the enhanced expression of Rae-1 on MC38 cells induced by 5-FU treatment is likely associated with the augmented tumoricidal activity of activated NK cells, cytotoxic activity was examined when signal transduction through NKG2D was inhibited with Abs neutralizing Rae-1 or NKG2D or both. Our finding that the cytotoxic activity of NK cells was not inhibited by either the anti–Rae-1 Ab or the anti-NKG2D Ab suggests that signal transduction through NKG2D is not required during the effector phase of tumor-cell killing by NK cells.

We analyzed the DC-induced cytotoxicity mediated by activated NK cells using other tumor cells as target. Interestingly, B16 melanoma cells were not sensitive to NK cells activated by DC-inoculation. Hepa1-6 hepatocellular carcinoma cells were sensitive to the activated NK cells, but augmentation of cytotoxic activity by 5-FU pretreatment was not observed. Although the mechanism of different responses is not clear, these difference might be caused by different NK cell-sensitivity and distinct pathway to induce apoptosis of these target cells.

However, results obtained from only in vitro/ex vivo methodology but not from in vivo studies are not sufficient to show the efficacy of this combined treatment. It was not easy to determine the optimal condition to obtain the synergistic effect by DCs and 5-FU in vivo. The problem was how to administer 5-FU to the mice with low and stable serum concentration of 5-FU. As intraperitoneal administration was not suitable for this purpose, we have tried to use an implantable infusion pump for lab animals set in the subcutaneous space or peritoneal cavity for 5-FU slow-releasing, which is similar to the continuous intra-arterial infusion of chemotherapeutic agents for human cancer treatment. Combined therapy with a DC vaccine and oral 5-FU, which achieves low, stable serum concentrations optimal for enhancing the sensitivity of NK cells for tumor cells while producing little immunosuppression, is a promising cancer treatment mediated by activated NK cells.

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