Effective Strategy of Dendritic Cell-based Immunotherapy for Advanced Tumor-bearing Hosts: the Critical Role of Th1-dominant Immunity[1]

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Abstract

Although dendritic cell (DC)-based cancer-specific immunotherapy is a potent strategy for various types of carcinomas, few clinical studies have yielded optimal antitumor effects. Systemic immunodeficiency is observed in patients with advanced malignant disease. In this study, we explored the ability to induce antitumor immunity of the cultured monocyte-derived DCs from hosts with advanced malignant disease, using a mouse model. We found remarkable dysfunction of DCs from mice with advanced cancer, which exhibited T helper (Th)2-dominant immunity, and subsequent reduced antitumor immune response. On the other hand, we found dramatic restoration of the ability of DCs to induce optimal antitumor immune responses after systemic administration of streptococcal preparation OK-432 to the tumor-bearing mice, which induced Th1-dominant immunity. In therapeutic experiments, intratumoral injections of immature DCs from the OK-432-treated mice, designated OK-DCs, enhanced inhibition of tumor growth compared with injections of immature DCs from mice with advanced malignancies, designated T-DCs ($P < 0.05$), leading to significant prolongation in overall survival ($P < 0.05$). In analysis of cell surface antigens, antigen-presenting capability and interleukin 12 production, we showed functional skewing in T-DCs and significant restorations in OK-DCs. More $CD8^+$ tumor-infiltrating lymphocytes were detected in the mice treated with OK-DCs; furthermore, CTL assays showed that intratumoral injection of OK-DCs induced tumor-specific immune response to spleen as great as those of N-DCs. These results suggested that Th1-dominant immunity might play a crucial role in the differentiation of DCs, and OK-432 might be useful for inducing optimal antitumor effects in DC-based immunotherapy in tumor-bearing hosts.

Introduction

$CD8^+$ T cells are an important effector arm in antitumor immunity, and DCs[3] are professional antigen-presenting cells that can elicit an effective T-cell response. In induction of antitumor immune responses, DCs play a multistep central role including: (*a*) capture and processing of tumor antigens; (*b*) immigration to regional lymph nodes; and (*c*) presentation of antigens and induction of antigen-specific T cells (1). In this regard, the use of DCs as a booster of antitumor response has been considered a promising strategy for cancer immunotherapy (2, 3). Several reports have described success in inducing tumor-specific immune responses after stimulation with tumor antigen-pulsed DCs *in vitro* and *in vivo* (4). We and others have reported that tumor-specific immunotherapy using DC targeting for tumor-associated antigens induced an effective antitumor immune response in patients with malignant disease (5–7). However, the apparent success in mice contrasts with the current states of clinical practice, in which only a minority of patients have thus far benefited from DC-based antitumor vaccination. One of the reasons for this appeared to be the immune dysfunction in patients with malignant diseases. In recent years, several studies have reported defective function of DCs in tumor-bearing mice and in cancer patients with advanced-stage disease (8–10). The major findings of these studies were low expression of cell surface antigens such as those of MHC class II, CD80, and CD86 in DCs derived from tumor-bearing hosts. This may be one of the key problems in eliminating the immune deficiency in cancer patients. Therefore, success in cancer immunotherapy requires overcoming the immunosuppression in tumor-bearing hosts and inducing optimal immune response to tumors.

OK-432 is a penicillin-inactivated and lyophilized preparation of the low-virulence strain Su of *Streptococcus pyogenes*. It is a biological response modifier and has been applied against various types of cancer because of its efficacy in enhancing antitumor immune response (11). Clini-

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[3] The abbreviations used are: DC, dendritic cell; NK, natural killer; IL, interleukin; Th, T helper; KE, Klinishche Einheit; mAb, monoclonal antibody; CM, culture medium; rm, recombinant murine; SEB, *Staphylococcal aureus* enterotoxin B; mAb, monoclonal antibody; PE, phycoerythrin; MLR, mixed lymphocyte reaction; CD40L, CD40 ligand; MCF, mean channel fluorescence; BrdUrd, 5-bromo-2-dexyuridine; PerCP, Peridinin chlorophyll protein.

cally, it has been used especially in Japan as an adjuvant therapy for cancer patients (12, 13). It has been reported that OK-432 augments the activity of neutrophils, macrophages, lymphocytes, and NK cells and induces multiple cytokines (14, 15). OK-432 was reported recently to be a potent inducer of IL-12 and a Th1-dominant state, which subsequently induced IFN-γ production in a mouse model (16). In addition, several studies have showed that Th1-type cytokines enhance the therapeutic efficacy of antitumor responses and that Th1-dominant immunity is superior to Th2-dominant immunity in the induction of antitumor immunity (17).

In this study, we demonstrated impairment of antitumor immune response induced by DCs derived from donors with advanced malignant tumor exhibiting Th2-dominant immunity. We also showed improvement of antitumor immune response elicited by DCs derived from these hosts that received a systemic administration of OK-432, which exhibited Th1-dominant immunity.

Materials and Methods

Mice. Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, 5–8 weeks of age, were purchased from SLC (Shizuoka, Japan) and used for all experiments at the age of 8–11 weeks. All animals were acclimated for at least 1 week before the experiments began.

Cell Lines. Colon26 murine colon adenocarcinoma, BALB/c syngeneic, was provided by the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). Colon26 clone 20 (Colon26-20), a subline of Colon26, was kindly provided by Nippon Roche Research Center, Kamakura, Japan (18). These cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol (Wako, Osaka, Japan).

Biological Response Modifier. OK-432 (Picibanil; kindly provided by Chugai Pharmaceutical Co., Tokyo, Japan) is a lyophilized preparation of group A streptococcus, type 3, Su strain, inactivated with penicillin G. The concentration of OK-432 is expressed units designated as in KE (clinical unit). One KE of OK-432 equals 0.1 mg of dried streptococci.

Preparation of DCs. Monocyte-derived DCs were generated as described previously (19). Briefly, peripheral blood was isolated from mice by cardiac puncture and diluted in HBSS containing heparin. Peripheral mononuclear cells were isolated by gradient centrifugation (Lympholyte M; Sanbio, Uden, the Netherlands), and contaminating lymphocytes were depleted with a mixture of anti-B220 (RA3–6B2), anti-CD4 (GK1.5), anti-CD8 mAbs (53.6.72; RA3–6B2 and GK1.5, kindly provided by the Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, and 53.6.72, from PharMingen, San Diego, CA) and rabbit complement (Cedarlane Laboratories Limited, Hornby, Ontario, Canada). The obtained cells were resuspended in CM consisting of RPMI 1640, supplemented with 10% FCS and 50 μM 2-mercaptoethanol and subjected to 24 h adherence at 37°C in 6-well plates at 10^7 cells/well. Nonadherent cells were removed by extensive washing, and adherent cells were cultured in CM containing recombinant murine granulocyte/macrophage-colony stimulating factor (1000 units/ml) and rmIL-4 (1000 units/ml; both from Pepro Tech EC, London, United Kingdom). After 8 days of culture, nonadherent cells were obtained as DCs, and the majority of these cells exhibited typical dendritic morphology and high expression of CD11c, a dendritic cell-specific surface marker, on flow cytometry analysis (purity, >80%).

Tumor-bearing and OK-432-treated Models. Colon26-20 cells (1×10^6) were inoculated s.c. in the flank of BALB/c mice. When these tumors reached 20 mm in diameter, the tumor-bearing mice were sacrificed for preparation of DCs derived from tumor-bearing mice, termed T-DCs. In the OK-432-treated model, OK-432 (1 KE/body) was injected i.p. every other day after the inoculation of Colon26-20. When tumors reached 20 mm in diameter, these mice were sacrificed for preparation of DCs derived from the tumor-bearing mice treated with OK-432, termed OK-DCs. For the control study, normal mice were sacrificed, and DCs were generated under the same conditions as T-DCs and OK-DCs, and termed N-DCs.

Analysis of Intracellular Cytokines Synthesis. For detection of intracellular cytokine synthesis using flow cytometry, we used methods reported previously (20). Briefly, spleen cells were obtained from sacrificed mice, suspended in culture medium at 10^6/ml, and stimulated with 2 μg/ml SEB (Sigma Chemical Co., St. Louis, MO), 100 IU/ml rmIL-2 (PeproTech EC LTD), and 100 IU/ml rmIL-4. After 6 days of SEB culture, harvested cells were restimulated with 5 ng/ml phorbol 12-myristate 13-acetate (Sigma), and 1 μg/ml ionomycin (Sigma) in the presence GolgiStop (PharMingen) according to the manufacturer's protocol (Cytofix/Cytoperm Plus Cytostain kit; PharMingen). For staining, we used the kit from PharMingen and followed the recommended protocol. The following mAbs were used: FITC-conjugated anti-mIFN-γ, PE-conjugated anti-mIL-4, PerCP-conjugated anti-CD4, and appropriate isotype controls (PharMingen). Ten thousand cells were collected for each sample using FACScan, and the data obtained were analyzed with CellQuest software (Becton Dickinson).

Analysis of Cell Surface Molecules. For phenotypic analysis of DCs, PE-conjugated mAbs against murine cell surface molecules (CD80, CD86, CD40, class I, class II, and appropriate isotype controls, all from PharMingen, San Diego, CA) were used, and cytometric analysis was performed using a FACScan (Becton Dickinson, San Jose, CA). FITC-conjugated anti-CD11c mAb (PharMingen) was used to gate on the CD11c bright population of DCs. Ten thousand cells were collected for each sample, and the data obtained were analyzed with CellQuest software. Fc receptors were blocked by the anti-Fc receptor antibody (2.4G2, kindly provided by the Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) before staining of cell surface molecules.

Endocytosis Assay. The temperature-dependent uptake of FITC-labeled dextran was used to measure endocytotic function according to a modification of the procedure described previously (21). Fresh DCs were suspended in 0.5 ml of CM and cultured with FITC-labeled dextran (Sigma) for 60 min at either 0°C or 37°C. Cells expressing CD11c were

stained by adding anti-CD11c-PE during the last 5 min of the assay; the reaction was terminated by adding ice-cold PBS containing 0.1% azide. The stained cells were washed three times, and the $CD11c^+$ population was analyzed immediately for the intracellular accumulation of the FITC label by cytometric analysis. The degree of endocytosis was determined by comparing the intracellular uptake at 37°C with the nonspecific binding that occurred at 0°C.

MLR. MLR was carried out by coculturing C57BL/6 mouse spleen cells (5×10^6) as responder cells with various numbers of mitomycin C (Kyowa Hakko Co. Ltd., Osaka, Japan)-treated, BALB/c-derived DCs as effector cells. After 5 days of coculture, the proliferation of responder cells was estimated by Cell Proliferation ELISA BrdUrd (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer's protocol (22).

Cytokine Production Assay. Serum was collected when the mice were sacrificed to obtain peripheral blood. Serum cytokine levels (IL-4, IL-6, IFN-γ, and TNF-α) were measured by mouse ELISA kits (BioSource, Camarillo, CA). For investigation of IL-12 production, the harvested DCs (5×10^6/ml) were resuspended in CM containing CD40L (5 μg/ml; PharMingen) and cultured for 72 h. Supernatants of the culture were collected, and the quantity of secreted IL-12 (p40/p70) was determined by ELISA (COSMO bio, Tokyo, Japan). This ELISA kit could detect both p70 IL-12 and p40 IL-12.

Tumor Treatment Models. Mice were generally inoculated intradermally in the shaved flanks bilaterally with 5×10^5 Colon26 cells on day 0. On day 5, when tumor size reached approximately 20–40 mm^3, 1×10^7 DCs (N-DCs, T-DCs, and OK-DCs) suspended in HBSS were injected in the left flank intratumorally. On day 7, 1×10^7 DCs were injected in the left tumor again. Tumor growth was monitored every other day by measuring two perpendicular tumor diameters and tumor height with calipers. For comparison with the DC therapy group, mice of the nontherapy group were injected with HBSS.

Immunohistochemistry. Tumors were resected on the 7th day after treatment with DCs and were embedded in OCT compound (Tissue Tek; Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. Cryostat sections 6-μm thick were thaw-mounted on slides, air-dried, and stored desiccated at −20°C. Sections were fixed for 15 min in cold acetone, hydrated in PBS, and incubated in protein-blocking solution (Block Ace; Dainihon Seiyaku, Osaka, Japan) for 30 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. To detect murine $CD8^+$ T cells, the sections were then incubated for 1 h with anti-CD8α mAb (53.6.72; PharMingen). After being washed in PBS, they were incubated with biotin-labeled antirat IgG (Vector Laboratories, Burlingame, CA) and then subjected to treatment with avidin-biotin-peroxidase complex using a DAKO LSAB Peroxidase kit (DAKO, Carpinteria, CA). The color reaction was developed in 3,3′-diaminobenzidine solution, and counterstaining was performed with Mayer's hematoxylin solution.

CTL Assays. Splenocytes were harvested from two mice/group 7 days after intratumoral injection with DCs. These cells (2×10^6) were restimulated *in vitro* with 2×10^5 mitomycin C-treated Colon26 cells in the presence of rmIL-2 (25 IU/ml). Five days later, restimulated cells were used as effectors for the standard 4-h ^{51}Cr release assay against Colon26, YAC-1, and Meth A, a syngeneic tumor cell. In brief, target cells (1×10^6 of each) were labeled with 100 μCi of $Na_2\,^{51}CrO_4$ for 1 h. After washing twice, these effector and target cells were placed at an appropriate E:T ratio in 96-well, round-bottomed palates. The supernatant (100 μl) was collected after 4-h incubation, and the radioactivity was counted in a gamma counter. The percentage of the specific lysis was calculated using the following formula: % specific lysis = 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release).

Statistical Analysis. The significance of differences between groups was analyzed using the *t* test and repeated-measures ANOVA. Survival analysis was performed with the Kaplan-Meier test. The level of significance was set at $P < 0.05$.

Results

Colon26-20 Induced Weight Loss and a Th2-dominant State in Tumor-bearing Mice. To elucidate the characteristics of the Colon26-20-bearing mice, we first estimated the ability of the tumors to induce abnormalities associated with weight loss and serum cytokine level. Fig. 1 shows the carcass weights and serum cytokines levels (IFN-γ, TNF-α, IL-4, and IL-6) determined when mice were sacrificed to obtain DCs. Colon26-20 induced significant weight loss ($P < 0.01$) and significant elevations of serum Th2 type cytokines such as IL-4 and IL-6 ($P < 0.01$, $P < 0.001$, respectively) in tumor-bearing mice compared with normal mice.

Systemic Administration of OK-432 Induced a Th1-dominant State in Colon26-20 Tumor-bearing Mice. In OK-432-treated tumor-bearing mice, a significantly high level of IFN-γ was observed ($P < 0.001$), and an elevation of Th2-type cytokine levels, such as IL-4 and IL-6, were suppressed ($P < 0.01$ and $P < 0.05$, respectively; Fig. 1, *B, C,* and *D*) To estimate systemic immunity in normal mice, tumor-bearing mice, and OK-432-treated tumor-bearing mice, splenocytes were obtained from each group, and the intracellular cytokine synthesis of $CD4^+$ T lymphocytes was determined by flow cytometric analysis (Fig. 1*F*). T cells were activated and stained by PerCP-conjugated, anti-CD4; FITC-conjugated, anti-IFN-γ; and PE-conjugated, anti-IL-4 antibodies. $CD4^+$ cells from tumor-bearing mice were stained in the IL-4 (Th2-type cytokine)-dominant sate (IFN-γ:IL-4 ratio, 0.5), whereas $CD4^+$ cells from normal and OK-432-treated tumor-bearing mice were stained in the IFN-γ (Th1-type cytokine)-dominant state (IFN-γ:IL-4 ratios, 5.0 and 6.2, respectively).

Phenotypic Differences of Three Distinct DC Subsets (N-DCs, T-DCs, and OK-DCs). DCs from the three groups (normal hosts, tumor-bearing hosts, and OK-432-treated tumor-bearing hosts) were generated by 8 days of culture under the same conditions as described in "Materials and Methods." The DCs derived from these groups were designated N-DCs, T-DCs, and OK-DCs, respectively. To investigate expression of cell surface antigens, these three DCs were examined by flow cytometric analysis. Fig. 2 shows MCF intensities of each antigen. Significantly low expression

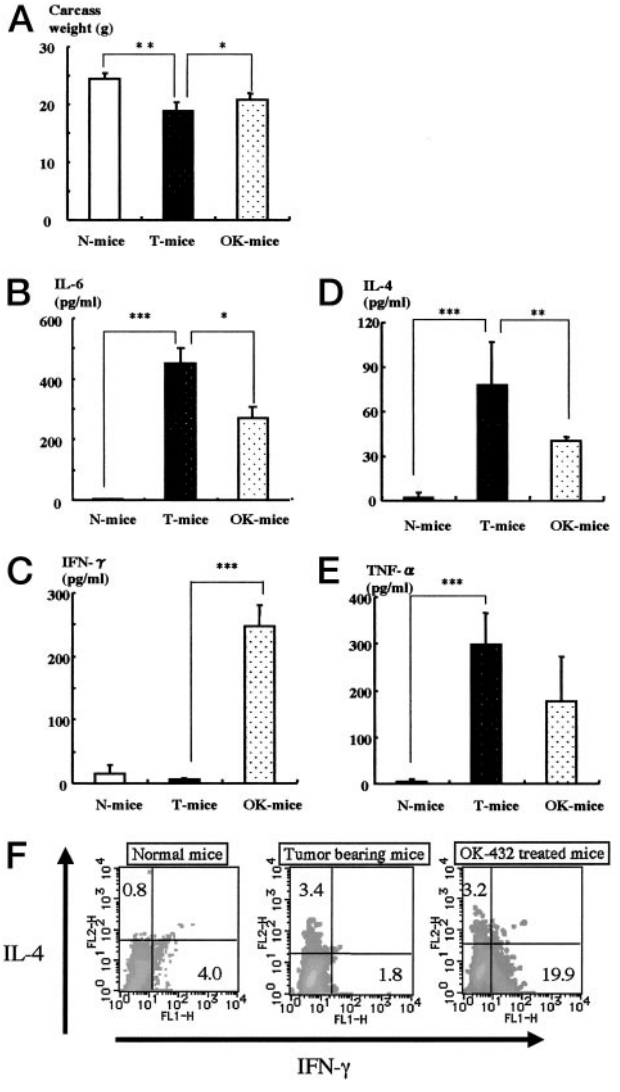

Fig. 1. Carcass weight and serum cytokine levels of normal, Colon26-20-bearing and OK-432-treated Colon26-20-bearing mice, designated as *N-mice, T-mice,* and *OK-mice,* respectively. When mice were sacrificed to obtain DCs, carcass weights were determined after resection of tumors (*A*). At the same time, serum was collected from the mice, and serum cytokine (IL-4, IL-6, IFN-γ, and TNF-α) levels were measured by ELISA (*B–E*). Each group consisted of six mice. In Colon26-20-bearing mice, significant weight loss and significant high serum IL-6 levels were found compared with those in normal and OK-432-treated mice (*A* and *B*). A high level of IFN-γ was observed in OK-432-treated tumor-bearing mice (*C*). In the tumor-bearing mice, a significant high level of IL-4 and TNF-α was also observed as compared with that of normal control mice (*D* and *E*). $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$. *Bars,* SD. *F,* intracellular cytokine staining of spleen cells of the mice from the three different groups. Spleen cells were obtained when mice were sacrificed to obtain DCs, suspended in culture medium at 10^6/ml, and stimulated with SEB, rmIL-2, and rmIL-4. After 6 days of SEB culture, harvested cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence GolgiStop for 3 h. For staining, the following mAbs were used: FITC-conjugated anti-mIFN-γ; PE-conjugated anti-mIL-4; PerCP-conjugated anti-CD4; and appropriate isotype controls. Ten thousand $CD4^+$ cells were collected for each sample, and cytoplasmic cytokine expression was determined by flow cytometric analysis. The numbers show percentages of Th1 (IFN-γ)-or Th2 (IL-4)-positive cells. Systemic administration of OK-432 induced Th1-dominant states in Colon26-20-bearing mice. In contrast, the tumor-bearing mice were in Th2-dominant states. Results are representative of three separate experiments.

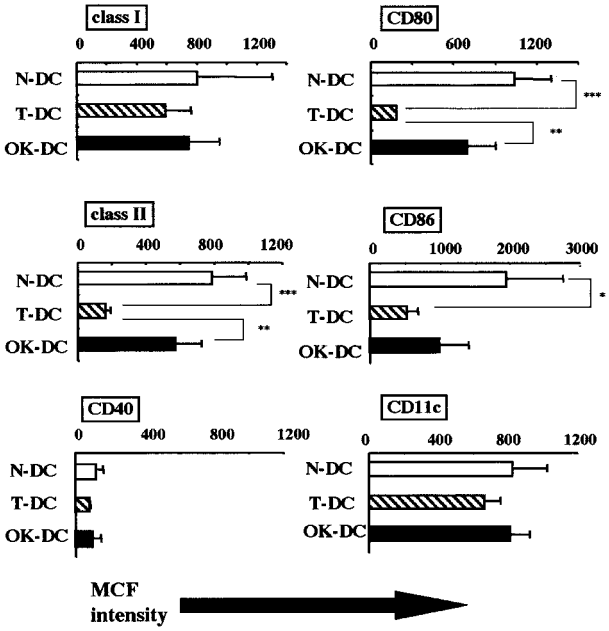

Fig. 2. Phenotypic examination of N-DCs, T-DCs, and OK-DCs using flow cytometric analysis. DCs were stained with mAbs as described in "Materials and Methods," and MCF intensities were compared among the DCs from the three different groups of mice. A significantly low expression of class II, CD80, and CD86 was detected in T-DCs compared with N-DCs. In OK-DCs, a significantly high expression of class II and CD80 was detected. FITC-conjugated anti-CD11c mAb was used to gate on the CD11c bright population of DCs. All of these cells could be considered immature DCs, because they had a high expression of CD11c and a low level expression of CD40. Ten thousand cells were collected for each sample, and the data were analyzed with CellQuest software. Results were given as means; *bars,* SD. Similar results were obtained for four experiments. $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.005$.

of class II, CD80, and CD86 was detected in T-DCs compared with N-DCs, whereas in OK-DCs, significantly high expression of class II and CD80 were detected, indicating that the cell surface antigen expressions of OK-DCs were nearly equal to those of N-DCs. All of these cells could be considered immature DCs, because they had the morphological characteristics of immature DCs, high level of expression of CD11c and low level expression of CD40.

Functional Differences of Three Distinct DC Subsets (N-DCs, T-DCs, and OK-DCs). For the induction of therapeutic antitumor immunity, it is the first important step for DCs to capture tumor antigens. To estimate the ability to endocytose of the three DC subsets, we performed endocytosis assay using flow cytometry. As shown in Fig. 3*A,* high fluorescence intensities were observed in all of the subsets, and there was no significant difference among them. These results suggested that there was no difference in the capability to capture antigens, and the results also supported that all of the DC subsets belong to immature DCs. To compare antigen presentation properties of DCs, we next performed allogeneic MLR. Using C57BL/6 mouse splenocytes (5×10^6) as responder cells, MLR was performed by coculturing such cells with various numbers of mitomycin C-treated DCs derived from each group of BALB/c mice. The cell proliferation of the responders was estimated by BrdUrd uptake

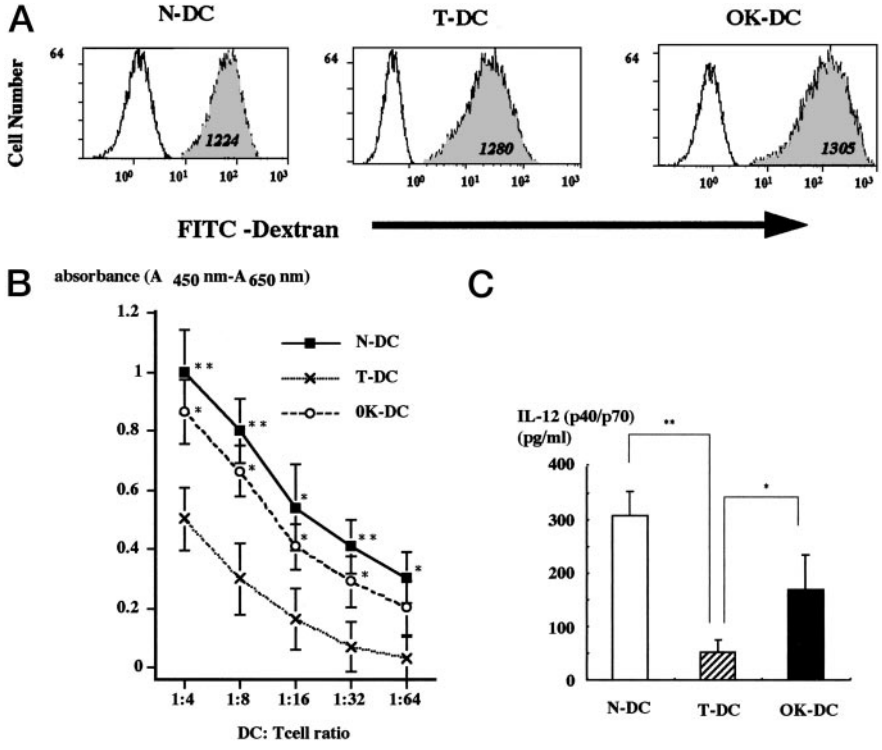

Fig. 3. *A,* FITC-dextran uptake of N-DCs, T-DCs, and OK-DCs. There was no significant difference in the capability to capture antigens. The results also supported that all of these cells could be considered immature DCs, which exhibited the ability to endocytose. The three DC subsets were cultured at either 0°C (*white histograms*) or 37°C (*black histograms*) for 90 min in the presence of FITC-labeled dextran (1 mg/ml). Cells were counterstained with CD11c-PE, and the intracellular accumulation of FITC-dextran on the $CD11c^+$ population was determined by cytometric analysis. Numbers indicate relative MCF intensities for the FITC label at 37°C minus that at 0°C. Similar results were obtained for three experiments, and representative results are shown. *B,* stimulating activity of DCs in allogeneic MLR. The DCs from Colon26-20-bearing mice (T-DCs) exhibited an inferior capacity to generate alloreactivity, whereas the DCs from OK-432-treated mice (OK-DCs) exhibited superior stimulating activity. Mitomycin C-treated DCs from three groups of BALB/c mice ($H\text{-}2^d$) were assessed in MLR with splenocytes from C57BL/6 ($H\text{-}2^b$) as responders. The responder cell numbers were kept constant, and the DC numbers were varied. After 5-day coculture, T-cell proliferation was determined by BrdUrd uptake. Results were given as means of triplicate cultures; *bars,* SD. Similar results were obtained for three experiments. *, $P < 0.05$; **, $P < 0.01$ for N-DCs or OK-DCs *versus* T-DCs. *C,* IL-12 (p40 p70) production by DCs. The capability of OK-DCs to produce IL-12 was significantly higher than that of T-DCs ($P < 0.05$). N-DCs, T-DCs, and OK-DCs were incubated in culture medium with CD40L for 72 h, and IL-12 secreted in the supernatants was quantified by cytokine-specific IL-12 ELISA. Results are given as means of triplicate cultures; *bars,* SD. Similar results were obtained for three experiments. *, $P < 0.05$; **, $P < 0.01$.

using ELISA. Compared with T-DCs, both N-DCs and OK-DCs had markedly increased capacity to generate MLR with allogeneic C57BL/6 responder lymphocytes (Fig. 3*B*). IL-12 plays a key role in the induction of antitumor response including activation of T cells and NK cells (23). To determine the capabilities of the three DC subsets to produce IL-12, DCs were stimulated with CD40L for 72 h, and IL-12 secreted in the supernatants was quantified by cytokine-specific IL-12 ELISA (Fig. 3*C*). As shown in Fig. 3*C,* a significant decrease in ability to produce IL-12 (p40/p70) was detected in T-DCs compared with N-DCs ($P < 0.01$). The capability of OK-DCs to produce IL-12 was significantly higher than that of T-DCs ($P < 0.05$).

Inhibition of Tumor Growth Induced by Intratumoral Injection of N-DCs, T-DCs, and OK-DCs. Several studies have reported that direct intratumoral injections of immature DCs elicit effective cellular immunity to the tumor via a $CD8^+$ T cell-dependent mechanism (23, 24). To examine the antitumor effect of intratumoral injection with DCs, 1×10^7 N-DCs, T-DCs, or OK-DCs were injected into established Colon26 tumors on days 5 and 7 after tumor cell inoculation. As shown in Fig. 4*A,* N-DCs suppressed the growth of DC-treated tumors more significantly than did T-DCs ($P < 0.05$). OK-DCs significantly suppressed tumor growth compared with T-DCs ($P < 0.05$). To confirm the induction of systemic and therapeutic immunity, we examined the growth of the contralateral nontreated tumors, which were distant from the DC-treated tumors. As shown in Fig. 4*B,* significant suppression of tumor growth induced after intratumoral injections of N-DCs and OK-DCs was also observed in the noninjected tumors, compared with injection of T-DCs ($P < 0.05$ and $P < 0.05$, respectively). Next, to determine the therapeutic impact of systemic administration of OK-432 in tumor-bearing mice, we observed the prognosis of 12 mice treated with N-DCs, T-DCs, and OK-DCs. The 50-day survival rates after tumor inoculation were 75, 25, and 66%, respectively. The log-rank test revealed that treatment with N-DCs and OK-DCs prolonged survival significantly compared with treatment with T-DCs ($P < 0.05$ and $P < 0.05$, respectively; Fig. 4*C*).

Infiltration of $CD8^+$ Cells in Tumor Tissues Obtained from Treated Mice. To investigate effects of tumor-specific immunity, we resected the noninjected side tumors from

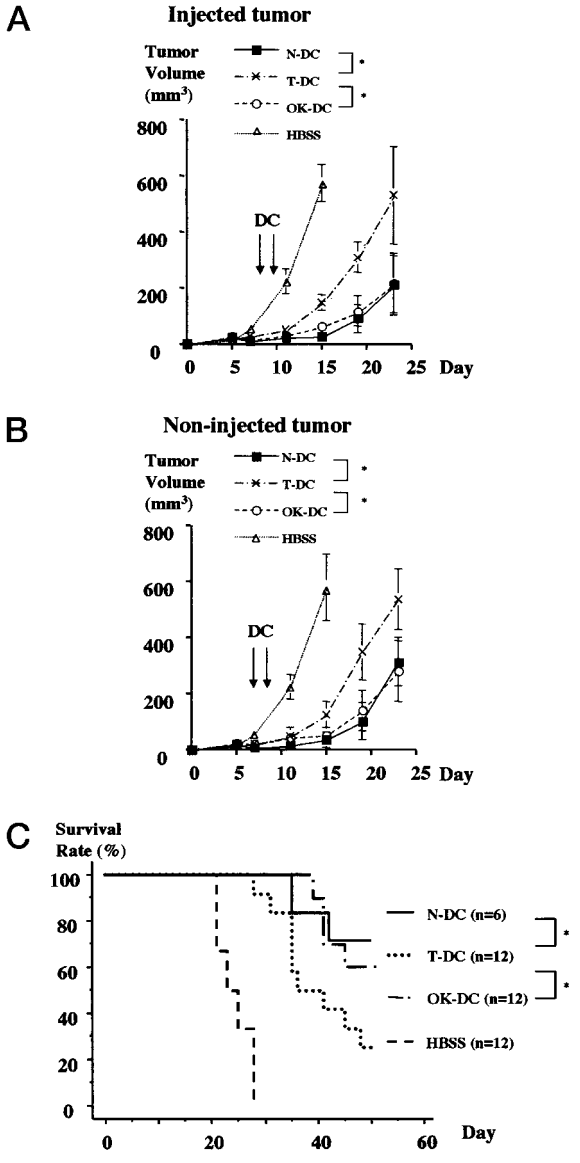

Fig. 4. *In vivo* antitumor effect induced by intratumoral injection of immature DCs. Direct intratumoral injections of OK-DCs yielded a restoration of antitumor effects observed using T-DCs. On day 0, BALB/c mice received 5×10^5 Colon26 cells in the flanks bilaterally and then received injections on days 5 and 7 with 1×10^7 immature DCs (N-DCs, T-DCs, and OK-DCs) suspended in HBSS and injected in the left flank intratumorally. A control group received HBSS alone. The size of tumors was recorded as tumor volume by measuring the largest perpendicular diameters and heights. *A,* injected side; *B,* noninjected side. Data are reported as the average tumor volume of 6–12 mice/group; *bars,* SD. The significance of differences between groups was analyzed using the repeated-measures ANOVA. ∗, $P < 0.05$. *C,* prognosis of the mice treated with intratumoral injections of immature DCs. Survival was monitored over time after tumor inoculation. Statistical analysis of the results by the log-rank test revealed that mice treated with N-DCs or OK-DCs had a significantly better prognosis than those treated with T-DCs. ∗, $P < 0.05$.

mice treated with the three different subsets of DCs on the 7th day after final injection of DCs and performed immunohistochemical staining with anti-CD8 mAb (Fig. 5, *A–C*). The mean number of immunoreactive cells in five different fields revealed significant suppression of CD8+ cell infiltration in

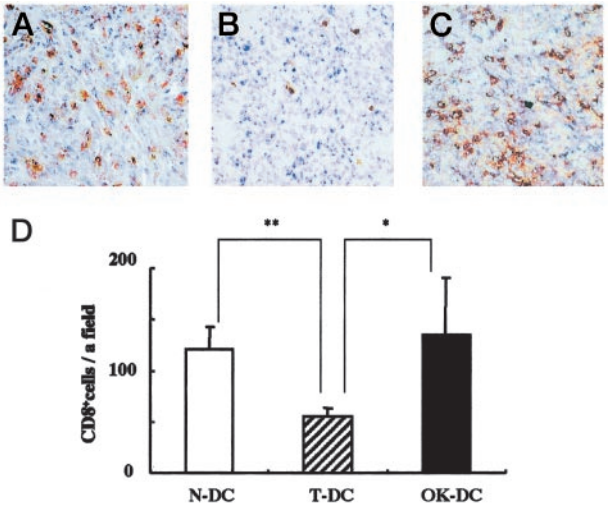

Fig. 5. Infiltration of $CD8^+$ cells in the noninjected tumors harvested from mice treated with DCs. The cryosections of tumor tissue in the noninjected side were stained with anti-CD8 mAb. More immunoreactive cells were detected in tumors obtained from mice treated with N-DCs (*A*) and OK-DCs (*C*) than from mice with tumors treated with T-DCs (*B*). Similar results were obtained for four experiments. *D,* mean numbers of $CD8^+$ cells infiltrating into noninjected tumor tissues were counted on five different fields at ×200. Significantly more CD8+ cells were observed in the tumors from the mice treated with N-DCs and OK-DCs than in the tumors from T-DC-treated mice. Results are given as means in five different fields; *bars,* SD. ∗, $P < 0.05$; ∗∗, $P < 0.005$.

the tumors treated with T-DCs compared with those treated with N-DCs or OK-DCs ($P < 0.005$ and $P < 0.05$, respectively; Fig. 5*D*). Treatment of tumors with OK-DCs restored infiltration of $CD8^+$ T cells as much as did treatment with N-DCs.

CTL Activity Induced by Intratumoral Injection of N-DCs, T-DCs, and OK-DCs. To confirm the induction of systemic and specific antitumor immunity, we examined the CTL activity of splenocytes from the mice treated with DCs. Splenocytes cultured with parental tumor cells for 5 days were tested for antitumor CTL and NK activity by 4-h ^{51}Cr-release assay. As shown in Fig. 6, intratumoral injection of N-DCs induced significant CTL activity against Colon26, as compared with injection of T-DCs. Treatment with OK-DCs induced significantly higher CTL activity than that of treatment with T-DCs. A low level of NK activity against YAC-1 cells was observed in both N-DC- and OK-DC-treated mice. No cytotoxic activity was observed against BALB/c syngeneic Meth-A tumor cells (data not shown). These results suggested that the CTL activity induced by treatment with OK-DCs appeared to be specific for Colon26 tumor.

Direct Effects of OK-432 to DCs: Analysis of Cell Surface Antigens. To examine direct effects of OK-432 to DCs, we analyzed the expression of cell surface antigens using flow cytometric analysis. Eight-day-cultured, monocyte-derived DCs from normal control mice were subsequent cultured with or without OK-432 (0.1 KE/ml) for 48-h (designated OK-432-treated DCs and control DCs, respectively). Each DC was harvested, and we analyzed the expression of cell surface antigens by flow cytometry. Table 1 shows the percentage of positively stained cells and MCF intensities of

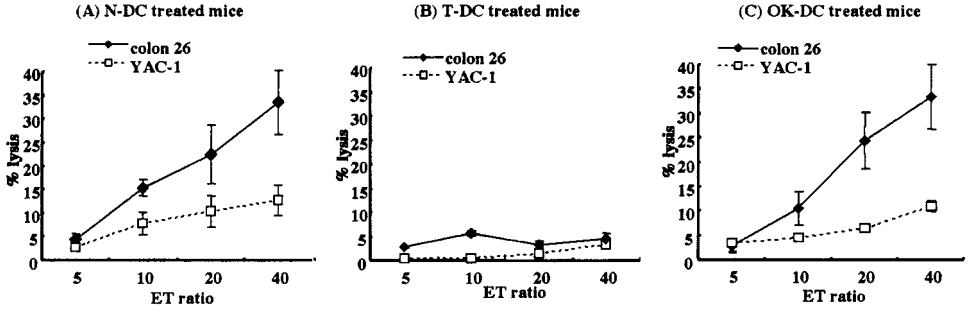

Fig. 6. Antitumor CTL activity detected in splenocytes harvested from mice treated with the three distinct DC subsets (N-DCs, T-DCs, and OK-DCs). Splenocytes from mice treated with N-DCs (*A*), T-DCs (*B*), and OK-DCs (*C*) were restimulated with mitomycin C-treated parental tumor cells and were tested 5 days later in a ^{51}Cr-release assay. Splenocytes from the mice treated with N-DCs showed significant CTL activity against Colon26, compared with T-DC-injected mice. OK-DCs induced significantly higher CTL activity than that of mice treated with T-DCs. A low level of NK activities against YAC-1 cells were observed in both N-DC- and OK-DC-treated mice. Similar results were obtained in three separate experiments. Data are presented as the means of triplicate samples; *bars,* SD.

Table 1 In vitro OK-432-treated DCs show modified surface phenotype[a]

Surface Antigen	% positive (MCF intensity)	
	Control DCs	OK-432-treated DCs
MHC class I	91.1 (1560)	98.6 (2267)
MHC class II	59.3 (753)	99.3 (1453)[b]
CD80	30.7 (725)	86.2 (1444)[b]
CD86	67.5 (2275)	99.4 (3401)
CD40	37.6 (397)	99.6 (1022)[b]

[a] Eight-day-cultured, monocyte-derived DCs were subsequently cultured with or without OK-432 (0.1 KE/ml) for 2 days, designated as "Control DCs" and "OK-432-treated DCs", respectively. After 2 days, DCs were harvested, and we analyzed the expression of cell surface antigens by flow cytometry. The table depicts the percentage of positively stained cells and MCF intensities. Results are representative of three separate experiments.
[b] $P < 0.05$.

each antigen. A significantly high expression of class II, CD80, and CD40 was detected in OK-432-treated DCs compared with that of control DCs. The similar tendency was observed using monocyte-derived DCs from tumor-bearing mice (data not shown).

Discussion

DC-based cancer immunotherapy has moved to the center stage in active immunotherapy (2, 3, 25). Adequate functioning of DCs is crucial for effective antitumor control and the success of cancer immunotherapy. The aim of this study was to determine whether the induction of Th1-dominant environment by systemic administrations of OK-432 to hosts with advanced malignant disease modulates the antitumor effect of DCs derived from the tumor-bearing hosts. In this study, we demonstrated successful biological modification of murine DCs with systemic administration of OK-432 to mice with advanced cancer. The following significant changes were found in DCs from the OK-432-treated tumor-bearing mice: (*a*) increased expression of MHC and costimulatory molecules; (*b*) increased antigen-presenting ability; and (*c*) increased ability to produce IL-12. Intratumoral injection of immature DCs from OK-432-treated mice thus enhanced induction of tumor-specific immunity, such as infiltration of $CD8^+$ cells into tumor tissues, and resulted in effective suppression of tumor growth.

Recent studies have demonstrated that the Th1/Th2 balance controlled by Th1 or Th2 cells producing cytokines plays important roles in various immune responses, including antitumor immunity (17). Th1 cells producing IFN-γ and IL-2 are essential for the induction of cellular and tumor immunity, whereas Th2 cells producing IL-4, IL-6, and IL-10 are associated with suppression of cytolytic activity (26–28). Several studies have shown increased levels of Th2 type cytokines in the peripheral blood of tumor-bearing mice or cancer patients (29, 30). In particular, great changes in cytokine environments and subsequent immune dysfunction have been demonstrated in advanced malignant disease (31, 32), and increases in serum level of IL-6 and TGF-β are reported to be related to progression of malignant disease (33). In this study, we found significantly high levels of serum IL-4, IL-6, and relative Th2-dominant state in our tumor-bearing mouse model. As a reason for the inferior functions of the DCs from the tumor-bearing state (T-DCs), it is possible that the changes in cytokine environment lead to down-regulation of the entire metabolism of progenitor cells of DCs in the tumor-bearing mice and subsequent suppression of antitumor cellular immunity via DCs. Another reason for the dysfunction of T-DCs involves tumor-derived factors. Several studies have reported many tumor-derived factors, which affect the differentiation of DCs, IL-6 (34, 35), IL-10 (36, 37), TGF-β (38), and VEGF (39), and some studies have reported defective maturation and skewing differentiation of DCs in tumor-bearing hosts (10, 40, 41). In this study, we used the cultured DCs but not DCs directly purified from the mice. These findings indicated that exposure of the progenitor cells of DCs in mice with advanced malignant disease to a Th2-type cytokine dominant environment and tumor-derived factors affected the differentiation of DCs.

Recent studies have reported that there is functional heterogeneity of DCs regulated by systemic immune systems (42), and differentiation of DCs is thought to be affected by the cytokine microenvironment (43). DCs in a Th1-type, cytokine-dominant environment provide optimal effects on antigen-specific CTLs, whereas in a Th2-dominant state,

such effects are suppressed (44). OK-432 is one of the biological response modifiers demonstrated to induce systemic Th1-dominant immunity *in vivo* and *in vitro* (16, 45). In the present study, as shown in Fig. 1, we successfully induced a Th1-dominant state in mouse models of advanced malignancy by repeated administration of OK-432, and we also restored the ability of the DCs from the OK-432-treated mice to induce optimal immune responses. These findings suggested that the induction of a Th1-dominant cytokine environment in tumor-bearing hosts with malignancies had a crucial role to elicit successful antitumor immune responses mediated by DCs.

OK-432 is a penicillin-killed streptococcal preparation, and it is reported to be a potent inducer of Th1-type cytokines (16). Recent studies have reported that a lipoteichoic acid-related molecule, designated OK-PSA, was isolated from OK-432 as a potent inducer of Th1-type cytokines (46). Moreover, OK-PSA was reported to be involved in toll-like receptors, which were expressed in myelomonocytic elements and play a fundamental role in pathogen recognition and activation of innate immunity (47, 48). It is possible that OK-PSA might have an effect on the progenitor cells of DCs, such as monocytes, in the OK-432-treated cachectic mice and improve their function to elicit antitumor immune responses.

Therefore, we propose the following possibilities in addressing the mechanism underlying our successful therapeutic model of mice with advanced malignant disease. In tumor-bearing mice, the progenitor cells of DCs were exposed to a Th2-biasing environment and tumor-derived suppressors such as IL-4 and IL-6; subsequently, these factors had an effect on the differentiation of progenitors, whereas in OK-432-treated tumor-bearing mice, repeated administration of OK-432 affected some kinds of immune cells such as T cells, NK cells, and macrophages, which induced a Th1-biasing environment around the progenitors of DCs. These cytokine environments differentiated the progenitors to potent DCs that were able to induce optimal antitumor immunity. Another possibility was proposed that OK-PSA, an effective component of OK-432, directly affected progenitor cells of DCs in myelomonocytic elements through toll-like receptor signaling and caused the differentiation of the cells suitable for an induction of cellular immunity to tumors. With an *in vitro* assay, we showed the progression of DC maturation in the presence of OK-432 on DC surface molecules (Table 1).

In consideration of the efficacy of tumor immunotherapy, the development of an efficient method to overcome immune dysfunction in tumor-bearing hosts is one of the most important factors in successful immunotherapy. For example, modification of DCs is required to enhance antitumor activity of DC-based immunotherapy in patients with malignant disease. Combined administration of DCs with cytokines, chemokines, biological response modifiers, or apoptosis-inducing agents has yielded encouraging results both *in vitro* and *in vivo* (24, 49–51). In addition, genetic modification of DCs to express tumor-associated antigens, cytokines, or chemokines has met with success in tumor treatment of preclinical animal models (23, 52, 53). We found that Th1-dominant immunity induced by systemic administration of OK-432 in tumor-bearing hosts is a rational strategy for DC-based cancer immunotherapy. On the basis of these findings, we are planning to apply for use of OK-432 in DC-based vaccination of cancer patients.

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