Biosciences) for 15 min at room temperature. Apropriate isotype controls were included for each sample.

2.5. Flow cytometric analysis

The samples were acquired on a FACSCalibur for four-color flow cytometry. Data analysis was performed using the CellQuest software (Becton Dickinson, CA, USA).

2.6. Statistical analysis

Data are indicated as means ± SD unless otherwise stated. The statistical significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric U test. $P < 0.05$ was considered significant.

3. Results

3.1. Frequencies of $CD4^+CD25^{high}$ T cells

To evaluate the frequencies of $CD4^+CD25^{high}$ T cell subsets that contain Tregs, MACS-sorted $CD4^+$ T cell subsets obtained from the patients with CH, LC and HCC and healthy controls were analyzed by flow cytometry following the staining with anti-CD4 and anti-CD25 monoclonal antibodies (Fig. 1A and B). Although the frequencies of $CD4^+CD25^{high}$ T cells were not changed in patients with CH, they were increased in patients with LC compared to the controls ($P < 0.05$). As reported, it is remarkably elevated in patients with HCC ($P < 0.0001$). The results indicated that $CD4^+CD25^{high}$ T cell subset containing Tregs are increased in patients complicated with liver malignancies.

3.2. Intracellular Foxp3 and cytokine production of the $CD4^+CD25^{high}$ T cell subset in HCC patients

The transcription factor Foxp3 is considered to be a specific marker for Tregs [22–24]. Intracellular Foxp3 levels were detected by using the specific mAb after the cell membrane permeabilization procedures (Fig. 2A). The percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was larger than that of $CD4^+CD25^{low/negative}$ subset, and it was also significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients (Fig. 2B). Thus, not only is the number of $CD4^+CD25^{high}$ T cells in HCC patients larger, but also the frequency of $Foxp3^+$ cells in HCC patients is higher than CH patient and healthy controls. This is consistent with previous reports of Tregs in patients with other malignancies.

Intracellular production of cytokines IL-4 and IL-10 of $CD4^+CD25^{high}$-$Foxp3^+$ T cell subset was quantitated following the stimulation with PMA/ionomycin using the specific mAbs by flow cytometry (Fig. 2C).

The levels of Th2 cytokines IL-4 and IL-10 were high in the $CD4^+CD25^{high}$ subsets. In addition, the levels of IL-4 and IL-10 were high in the $CD4^+CD25^{high}Foxp3^+$ T cell subset in HCC patient ($P < 0.005$) (Fig. 2D). These results suggest that the $CD4^+CD25^{high}Foxp3^+$ Treg subset in HCC patients may have a high potential to produce immunosuppressive cytokines.

3.3. Phenotypes of the $CD4^+CD25^{high}$ T cell subset in HCC patients

To determine the phenotypical properties of $CD4^+CD25^{high}$ T cell subset increased in patients with HCC, the expression levels of the seven reported surface molecules, CD45RA, CD45RO, CD62L, CCR7, CTLA-4, HLA-DR and GITR were quantitated by flow cytometry. Among the seven molecules, the proportions of $CD45RO^+$, $HLA\text{-}DR^+$ and $GITR^+$ cells were higher in the $CD4^+CD25^{high}$ T cell subset in all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$) (Fig. 3A and B). The percentage of $CD45RO^+$ cells in HCC patients were elevated compared to the patients with advanced liver diseases and healthy controls ($P < 0.01$). These data demonstrate that the $CD4^+CD25^{high}$ T cell subset highly expresses the surface molecule CD45RO in HCC patients, which may reflect the memory properties of T cells.

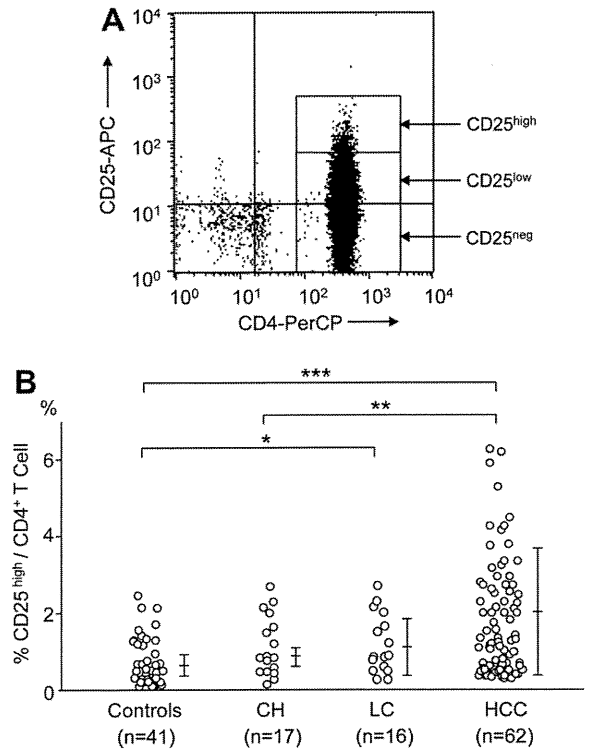

Fig. 1. Frequencies of $CD4^+CD25^{high}$ T cells in peripheral blood of HCC patients and controls. (A) Representative flow cytometric analysis of PBMCs (peripheral blood mononuclear cells) of an HCC patient. Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies as described in the Materials and Methods. (B) Percentages of $CD4^+CD25^{high}$ T cells in the peripheral blood of HCC ($n = 62$), LC ($n = 16$), CH ($n = 17$) patient, and healthy controls ($n = 41$). Percentages for individual patient analyzed are shown. The percentages represent the proportions of $CD4^+CD25^{high}$ T cells in total $CD4^+$ cells. The prevalence of $CD4^+CD25^{high}$ T cells in HCC patients was significantly higher than in healthy controls or CH patients. CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

3.4. $CD4^+CD25^{high}$ T Cell subset and dendritic cells of HCC patients

Several reports have suggested that the $CD4^+CD25^{high}$ T cell subset may interact with dendritic cells. To evaluate the frequencies of DCs in PBMC of HCC patients, whole blood cells were analyzed by flow cytometry following the staining with IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells "Plasmacytoid Subset" and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset". HCC patients were divided into two groups according to the frequencies of $CD45RO^{positive}$ cells in $CD4^+CD25^{high}$ T cell subsets ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained >83.8% positive cells in $CD4^+CD25^{high}$ T cells. The frequencies of $CD123^+$ plasmacytoid DCs were significantly higher in $CD45RO^{++}$, group ($P < 0.05$) (Fig. 5A and B), although those of $CD33^+$ myeloid DCs were not correlated with the subsets in $CD4^+CD25^{high}$ cells. These results showed that there are more tolerogenic plasmacytoid DCs in the PBMCs of HCC patients with higher frequencies of a memory subset of $CD4^+CD25^{high}$ T cells.

3.5. $CD4^+CD25^{high}$ T cell subset and tumor progression

To evaluate the association between $CD4^+CD25^{high}$ T cell phenotype and tumor progression, we compared the maximum tumor diameters, the number of tumors, tumor markers AFP (alpha-fetoprotein) and DCP (des-gamma-carboxyl prothrombin), TNM stages, Child-Pugh scores

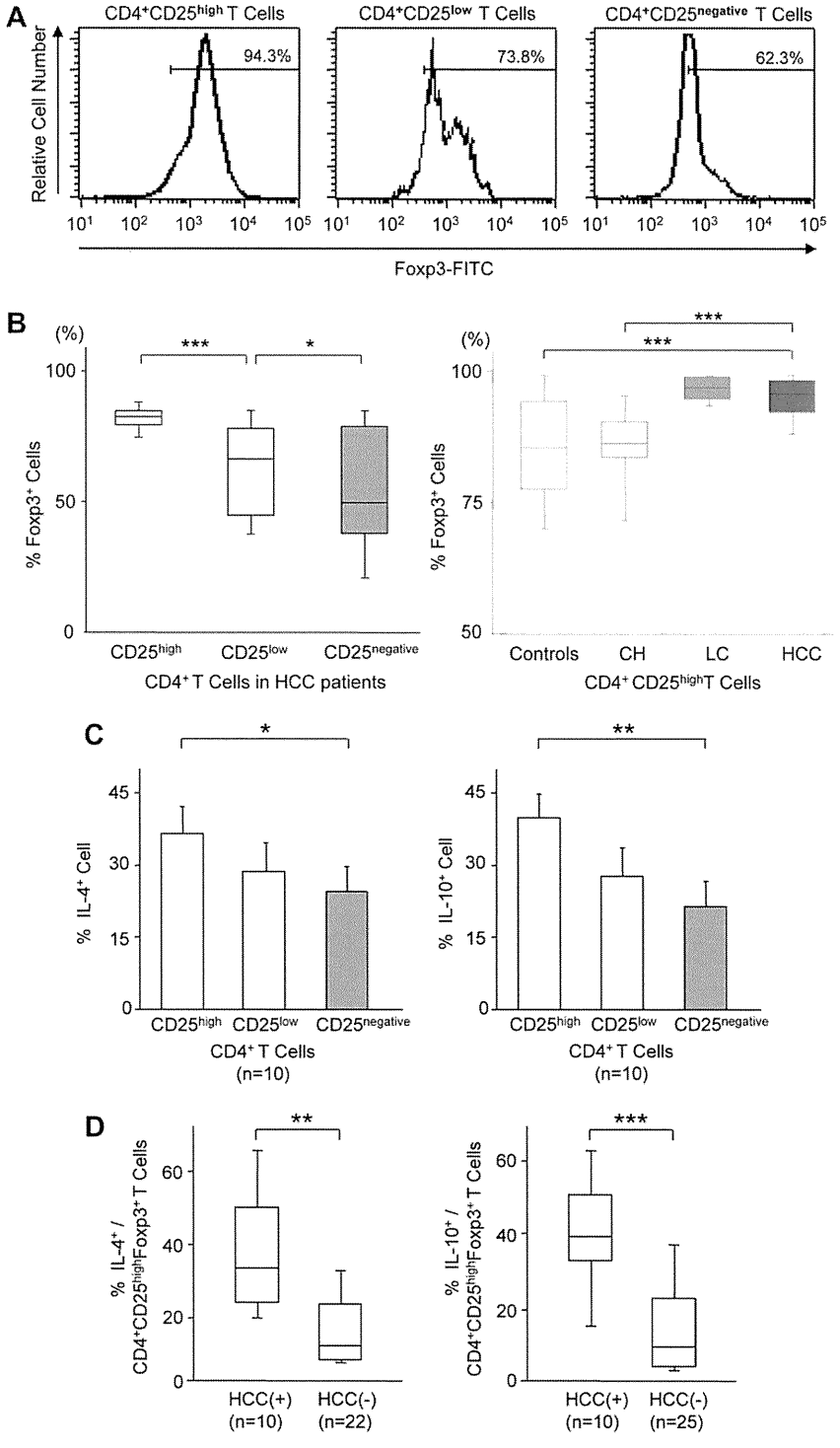

Fig. 2. Analysis of intracellular Foxp3 expression and cytokine production in $CD4^+$ $CD25^{high/low/negative}$ T cell subsets in HCC patients. (A) Representative expression of Foxp3 in $CD4^+$ T cells from an individual subject. Intracellular Foxp3 was stained following membrane permeabilization. Intracellular Foxp3 was detected by the specific mAb. (B) Statistical analysis in the left side panel shows that the percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{low/negative}$ T cell subsets, and in the right side panel shows that that of $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients. (C) Statistical analysis shows that the levels of Th2 cytokines IL-4 and IL-10 were remarkably high in the $CD4^+CD25^{high}$ T cell subset. (D) Comparison of intracellular cytokine production in $CD4^+CD25^{high}$ T cell subsets between patients with and without HCC. Healthy controls, patients with chronic hepatitis and liver cirrhosis were included in the HCC (−) column. IL-4 and IL-10 levels were higher in the $CD4^+CD25^{high}$ T cell subset in HCC patients. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

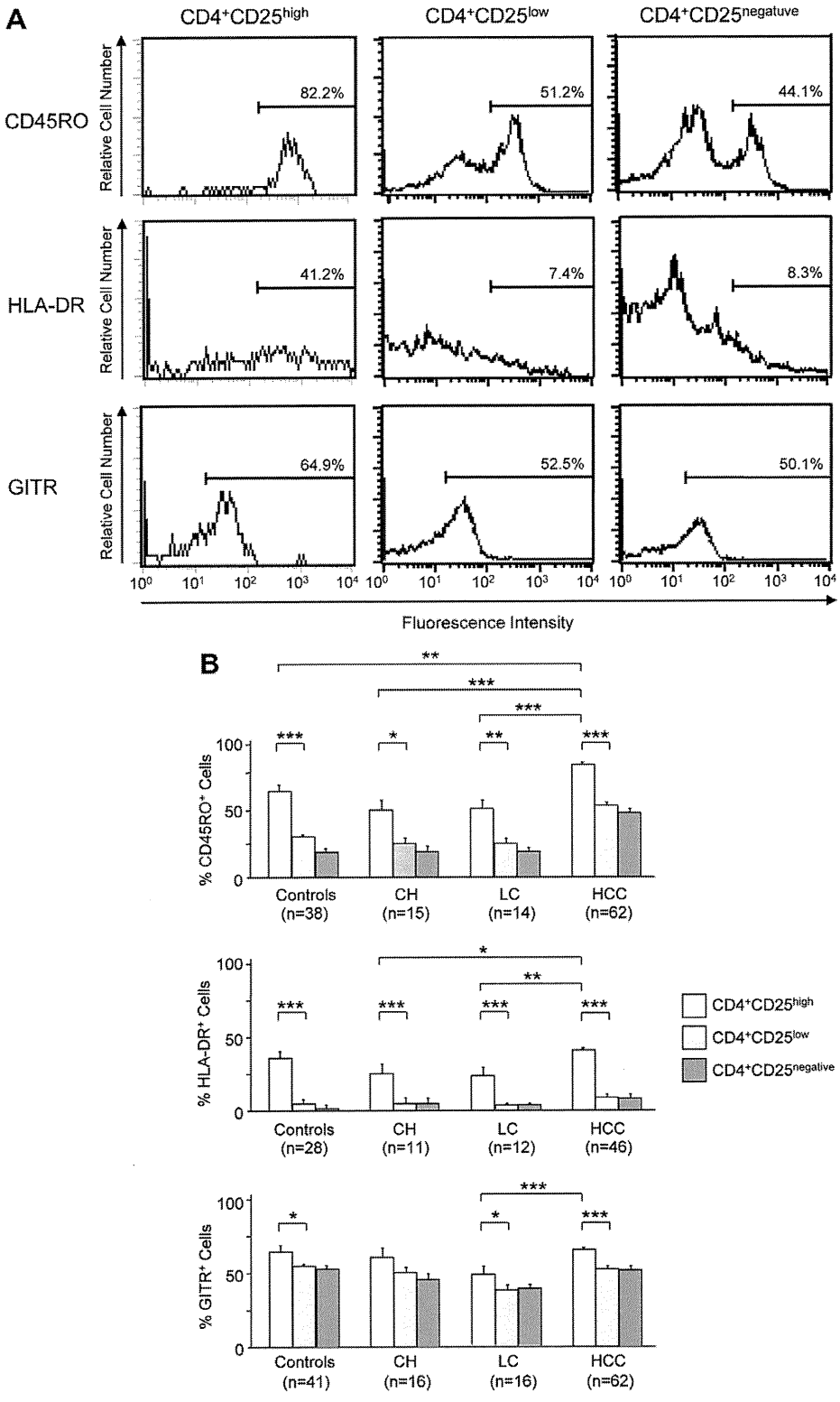

Fig. 3. Phenotypic analysis of $CD4^+CD25^{high/low/negative}$ T cell subsets in HCC patients. Fleshly isolated $CD4^+$ T cells (at least 2×10^5 cells/tube) from HCC patients were labeled with anti-CD4, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CCR7, anti-CTLA-4, anti-HLA-DR and anti-GITR mAbs. (A) Representative CD45RO, HLA-DR, and GITR expression profiles in $CD4^+$ T cell subsets that differ in CD25 expression. (B) Statistical analysis shows that the proportions of $CD45RO^+$, $HLA\text{-}DR^+$ and $GITR^+$ were elevated in the $CD4^+CD25^{high}$ T cell subsets of all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$). The percentage of $CD45RO^+$ cells in HCC patients was elevated compared to the patients with advanced liver diseases and healthy controls. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

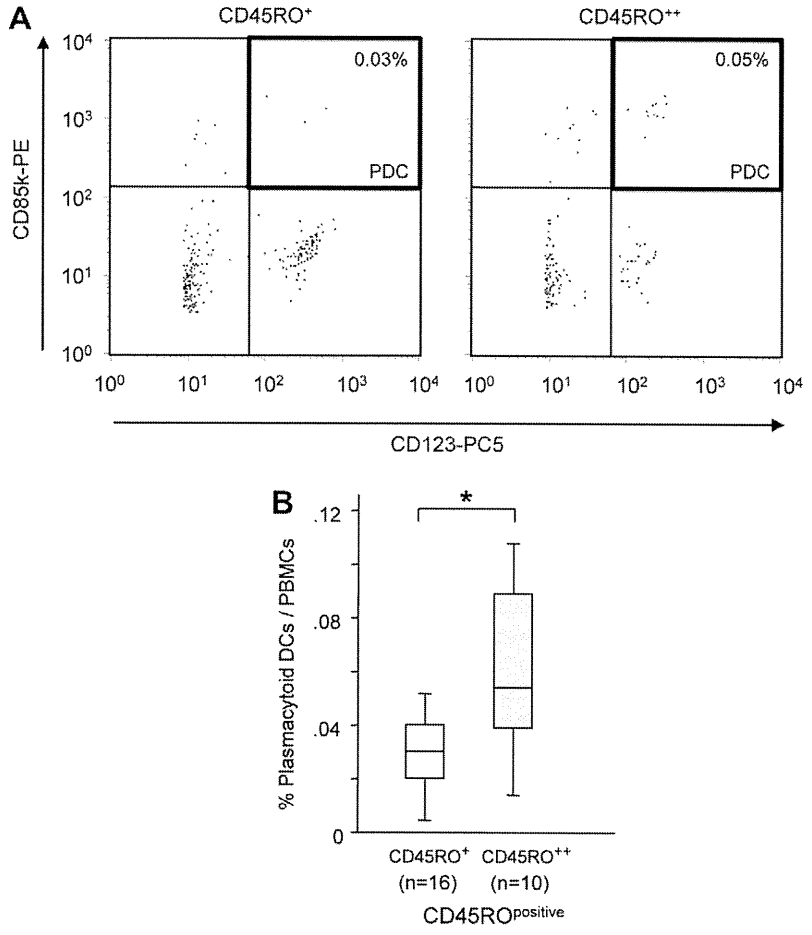

Fig. 4. Frequencies of plasmacytoid DCs in peripheral blood of HCC patients. Whole blood cells were analyzed by flow cytometry following staining with a combination of the mAbs. HCC patients were divided into two groups according to the frequencies of $CD45RO^{positivee}$ cells in $CD4^+CD25^{high}$ T cell subset ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained > 83.8% positive cells in $CD4^+CD25^{high}$ T cells. (A) Representative dot plots of plasmacytoid DCs. Plasmacytoid DCs of $CD45RO^+$ group are shown in the left panel and $CD45RO^{++}$ group in the right panel. (B) Statistical analysis shows that the frequencies of plasmacytoid DCs were significantly higher in $CD45RO^{++}$ group. *Indicates $P < 0.05$.

and fibrosis stages between two groups as described above. The levels of serum AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^+$ group (Fig. 4). Others were not significantly different between two groups. These results imply that a subset of Tregs may contribute to the progression of liver tumors.

4. Discussion

$CD4^+CD25^{high}$ $Foxp3^+$ regulatory T cells have been shown to increase in patients with malignancies to suppress the immune responses. In this study, we provide evidence that patients with HCC have increased frequencies of $CD4^+CD25^{high}$ T cells in their peripheral blood compared to healthy controls and chronic hepatitis patients. A large proportion of $CD4^+CD25^{high}$ T cells expressed Foxp3 and produced Th2 cytokines. We also showed that $CD4^+CD25^{high}$ T cells expressed high levels of CD45RO, HLA-DR and GITR, and, interestingly, the T cell frequencies expressing these surface molecules were associated with plasmacytoid DC numbers and maximum tumor diameters in HCC patients.

There are several reports of elevated numbers of Treg cells in the peripheral blood and tumor tissues of patients with different types of cancer [3–12]. The study of Unitt et al. provided the first report of increased $CD4^+CD25^+$ T cell frequency within tumor tissue compared to non-tumor tissue in HCC patients [13]. Ormandy et al. showed that the frequency of $CD4^+CD25^{high}$ T cells in peripheral blood of patients with HCC was significantly higher (3.92 ± 3.3%) than in healthy donors (1.17 ± 0.87%) and liver cirrhosis patients (0.78 ± 0.43%) [3]. Our data revealed that a minimal increase in $CD4^+CD25^{high}$ T cells was detected in LC patients and more pronounced changes were found in HCC patients.

We showed that higher percentages of $CD4^+CD25^{high}$ T cells produced Th2 cytokines IL-4 and IL-10 in HCC patients. Tregs were recently observed to produce IL-10 [25–27], which can be a major mediator of immune suppression [28–30]. Voo et al. reported that Tregs in the peripheral blood of healthy donors secreted IL-10 but not IL-2, IFN-γ, or IL-4 [31]. Schmitz-Winnenthal et al. demon-

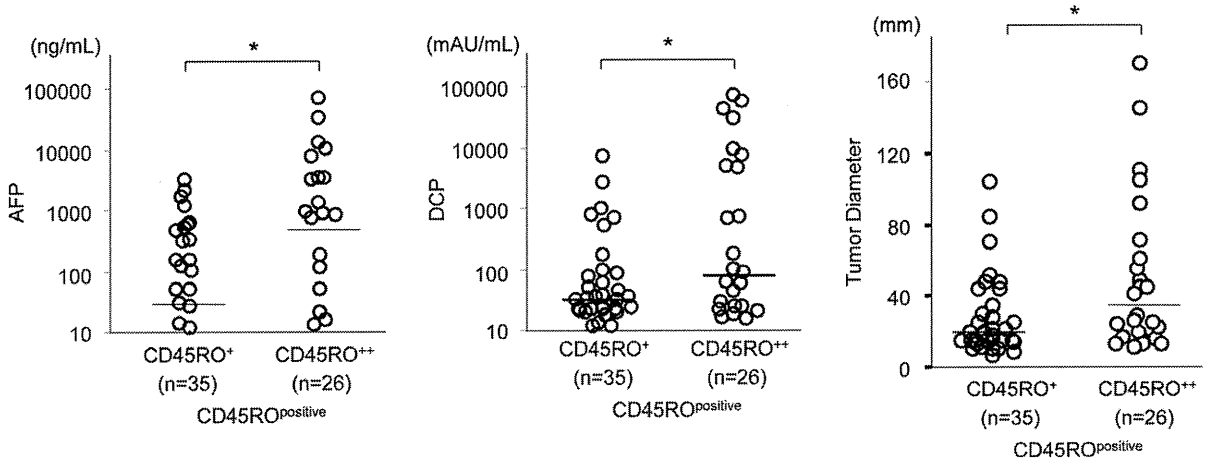

Fig. 5. Prevalence of $CD4^+CD25^{high/low/negative}$ T cell subsets and tumor progression. The levels of AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^+$ groups. AFP, alpha-fetoprotein; DCP, des-gamma-carboxyl prothrombin. *Indicates $P < 0.05$.

strated the presence of Treg secreting IL-10 but not IL-4 or IFN-γ upon antigen recognition in chronic pancreatitis patients [32]. The present data demonstrated that larger numbers of Tregs produced not only IL-10 but also IL-4 in HCC patients, which may contribute to the strong immunosuppressive properties of the T cells in liver malignancies.

It appears that Tregs consists of heterogenous populations within $CD4^+$ T cells, and that a subset of $CD4^+CD25^{high}$ T cells could be subdivided into different functional subsets based on the expression of various surface molecules [6]. The proportions of Tregs expressing these molecules are reported to be different in the various forms of cancer. The prevalence of $CD45RO^+$ and $GITR^+$ Treg cells is higher in $CD4^+CD25^{high}$ T cells than in $CD4^+CD25^{low/negative}$ T cells in renal cell carcinoma [4]. In head and neck squamous cell carcinoma, however, $CD4^+CD25^{high}$ T cells express CTLA-4, Foxp3, and CD62L but little GITR, and $CD25^{low/negative}$ T cells express intermediate to high levels of GITR and HLA-DR [8]. Our study showed that Tregs in HCC patients expressed significantly higher levels of CD45RO, HLA-DR and GITR compared to $CD4^+CD25^{low/negative}$ cells, suggesting that the activated populations of Tregs may contribute to the establishment of immunosuppressive microenvironments.

Little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated numbers of Treg cells in cancer. DCs have pivotal roles in the induction of tolerogenic/regulatory T cells [20,33]. In peripheral blood, there are two distinct populations of DCs which can be distinguished based on phenotypical and morphological characteristics; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [18,34]. Our data demonstrated that higher frequencies of $CD45RO^+CD4^+CD25^{high}$ T cells were associated with higher frequencies of pDCs in the peripheral blood of HCC patients. When the tumor antigens are assumed by pDCs through Toll-like receptor 9 (TLR9) via receptor-mediated endocytosis, secretions of pro-inflammatory cytokines, such as type I interferons (IFNs), would be caused. On the contrary, pDC may regulate anti-tumor immunity and support immune evasion and tumor escape. They exhibit reduced IFN-α production upon TLR9 stimulation and can induce IL-10 producing $CD4^+$ and $CD8^+$ Treg [35,36]. This suggests that anti-tumor immune responses can be regulated through both modulation of pDC function by the tumor and by limiting anti-tumor cytolytic activity through induction of $CD8^+$ Treg.

Concerning the association of Tregs and prognosis, it has been reported that an increased number of circulating Tregs predicts poor survival of patients with renal cell carcinoma [4], gastric and esophageal cancers [7], myelodysplastic syndrome [37] and HCC [11]. In addition, tumor-infiltrating Tregs were associated with reduced survival in ovarian cancer [12] and HCC patients [1]. In addition, we found that $CD45RO^+CD4^+CD25^{high}$ T cell subset was associated with larger tumor burdens, implying that a subset of Tregs may contribute to the promotion of tumor cell growth in the liver. However, it is also well possible that this just reflects stronger activation caused by a larger amount of antigen.

We performed the functional evaluation of Tregs derived from HCC patients by incubating with responder $CD4^+CD25^-$ T cells (Tresp). We observed that $CD45RO^+$ $CD4^+CD25^{high}$ T cells of HCC patients did not suppress the proliferation of responder T cells when co-cultured at Treg/Tresp ratios of 1:2 and 1:8 (data not shown). In contrast, Hoffmann et al. confirmed that the $CD45RA^+$ $CD4^+CD25^{high}$ T cells of healthy volunteers give rise to a homogeneous and highly suppressive Treg cell population, whereas $CD45RA\text{-}CD4^+CD25^{high}$ T cells generate cell lines with mixed phenotype and function [38]. Although the reasons of these conflicting data were not clarified in the current study, cell viability, apoptosis susceptibility, involvement of Th1 cytokines, and interaction to helper T cell subsets of Tregs obtained from HCC patients need to be evaluated in the future experiments.

This study may be helpful for a better characterization of Treg subsets in the peripheral circulation of patients with HCC, which may establish the immunosuppressive environment to promote tumor progression. Furthermore, to gain insights into changes in the Treg subsets

during the therapeutic option may lead to more effective immunotherapies against cancer and may improve prognosis.

Conflict of interest

None declared.

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BASIC RESEARCH

Cryoimmunologic Antitumor Effects Enhanced by Dendritic Cells in Osteosarcoma

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Abstract

Background We previously reported a limb-salvage technique by treating tumor-bearing bone with liquid nitrogen. We also reported systemic antitumor immunity was enhanced by cryotreatment in a murine osteosarcoma (LM8) model. We therefore combined the cryotreatment of tumor with dendritic cells to promote tumor-specific immune responses.

Questions/purposes We determined whether our technique could enhance systemic immune response and inhibit metastatic tumor growth in a murine osteosarcoma model.

Materials and Methods To evaluate activation of the immune response, we prepared six groups of C3H mice (80 mice total): (1) excision only, (2) dendritic cells without reimplantation of the cryotreated primary tumor, (3) reimplantation of the cryotreated primary tumor alone, (4) dendritic cells combined with reimplantation of the cryotreated primary tumor, (5) dendritic cells exposed to cryotreated tumor lysates without reimplantation of the cryotreated primary tumor, and (6) dendritic cells exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor. We then compared and verified the activation state of each group's antitumor immunity.

Results Mice that received dendritic cells exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor group had high serum interferon γ, reduced pulmonary metastases, and increased numbers of CD8(+) T lymphocytes in the metastatic areas.

Conclusions Combining tumor cryotreatment with dendritic cells enhanced systemic immune responses and inhibited metastatic tumor growth.

Clinical Relevance We suggest immunotherapy could be developed further to improve the treatment of osteosarcoma.

Introduction

The standard treatment of osteosarcoma consists of preoperative chemotherapy, surgical tumor excision, and postoperative chemotherapy. Limb-saving surgery is feasible in most cases. Advances in osteosarcoma treatment have now achieved a 5-year survival rate of 60% to 90% for patients, and limb function after reconstruction continues to improve with time [3, 16, 30, 46, 47, 49].

Tsuchiya et al. developed a new approach using frozen autografts [48] to improve reconstruction after osteosarcoma resection. The tumor is resected with an adequate margin, and the resected specimen is immersed in liquid

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Each author certifies that his or her institution has approved the animal protocol for this investigation, and that all investigations were conducted in conformity with ethical principles of research.
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nitrogen for 20 minutes to kill all tumor cells. After thawing, the specimen is returned to the original place with appropriate internal fixation to reconstruct the defect. Compared with heat-treated bones [8, 14], bone genetic proteins and native biomechanical structures are preserved after cryotreatment [53]. In one report limb function using the technique of Tsuchiya et al. was rated as excellent in 71.4% of patients, and good in 10.7%, as assessed by the functional evaluation system of Enneking [11]. Two studies suggest the approach enhanced bone formation when compared histologically with pasteurized bone and irradiated bone [43, 48]. Another advantage in reimplanting cryotreated tumor tissue is its effect on the immune system [50]: tumor tissue after cryoablation in situ provokes an immune reaction in patients with breast and prostate cancer [6, 8, 39]. Brewer et al. reported metastatic tumors sometimes disappear or shrink after in situ cryoablation of the primary tumor with liquid nitrogen [4]. The structure of tumor antigens is retained in frozen tumor, and leukocytes probably can recognize these antigens. Similar antitumor effects can be expected from our reconstructive procedure of reimplanting tumor-bearing bone after cryotreatment with liquid nitrogen.

Nishida et al. observed an inadequate antitumor effect after reimplantation of frozen tumor tissue alone [35]. However, the antitumor effect was enhanced by promoting nonspecific immune activation by intraperitoneal injection of OK-432, a substance extracted from alpha-Streptococcus pyogenes. This approach promotes inflammation and activation of dendritic cells (DCs) that initiate the specific antitumor effect [19]. This type of immunotherapy reportedly is effective for breast and prostate cancers [6, 8, 39]. Many groups have reported successful immunotherapy for osteosarcoma [5, 15, 18, 20, 22, 24, 25, 33, 34, 36, 42, 51, 52]. However, the ability to control metastatic lesions and local recurrence does not appear to be superior to other adjuvant treatments [2, 7, 13, 23, 29].

We therefore wondered whether combining cryotreatment and immunotherapy might enhance tumor response. We specifically determined whether: (1) antitumor immunity could be enhanced through activation and transfer of DCs combined with reimplantation of the cryotreated primary tumor, and (2) metastatic lesions could be prevented owing to the involvement of T lymphocytes in a murine osteosarcoma model (LM8).

Materials and Methods

Using a reported method to induce osteosarcoma [1, 35], we hypodermically implanted 1×10^6 LM8 cells (a murine osteosarcoma cell line) into the subcutaneous gluteal region of 80 female C3H mice, 6 to 8 weeks old. Tumors developed in all animals. Two weeks after inoculation, we surgically excised the tumors and cryotreated them with liquid nitrogen. We established the following six groups (Fig. 1): (1) the tumor was excised with wide margins 14 days after inoculation ($n = 15$); (2) the tumor was excised with wide margins 14 days after inoculation and bone marrow-derived DCs then were injected into the contralateral subcutaneous gluteal region without reimplantation of the cryotreated primary tumor twice a week ($n = 15$); (3) the tumor was excised with wide margins 14 days after inoculation and reimplanted after cryotreatment with liquid nitrogen into the contralateral gluteal region to evaluate for local recurrence from frozen tumor tissue ($n = 15$); (4) the tumor was excised 14 days after inoculation and reimplanted after cryotreatment into the contralateral gluteal region to evaluate for local recurrence, and DCs then were injected twice a week into this secondary site ($n = 15$); (5) the tumor was excised with wide margins 14 days after inoculation and DCs exposed to cryotreated tumor lysates were injected twice a week into the contralateral gluteal region without reimplantation of the cryotreated primary tumor ($n = 15$); and (6) the tumor was excised with wide margins 14 days after inoculation and reimplanted after the treatment with liquid nitrogen into the contralateral gluteal region to evaluate for local recurrence (same as Group 3) with the addition of DCs exposed to cryotreated tumor lysates injected twice a week ($n = 15$). We harvested tumor from 30 mice, and then the tumor was treated with liquid nitrogen to create the lysates. We presumed a systemic immune response would be induced by injecting DCs around the frozen tumor tissue. We microscopically determined the presence of metastases in the lungs 2 weeks after the tumor inoculation. We had previously confirmed the presence of pulmonary metastases in an additional 20 mice in a preliminary experiment in advance. We also confirmed that there were no viable cells after cryotreatment using liquid nitrogen, in agreement with a previous study [35]. We observed no recurrence of the tumor at the primary site of inoculation after excision. All experiments were performed under the guidelines for animal experiments as stipulated by the Kanazawa University Graduate School of Medical Science [37].

LM8 cells, derived from Dunn osteosarcoma, were provided by the Riken BioResource Center (Saitama, Japan). The cells were maintained in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 μg streptomycin per mL, and 100 units penicillin per mL and were cultured at 37°C in 5% CO_2. To establish local implantation of the tumor and subsequent lung metastasis, the LM8 cells (1×10^6) were suspended in 0.2 mL phosphate-buffered saline (PBS) and subcutaneously inoculated into the right

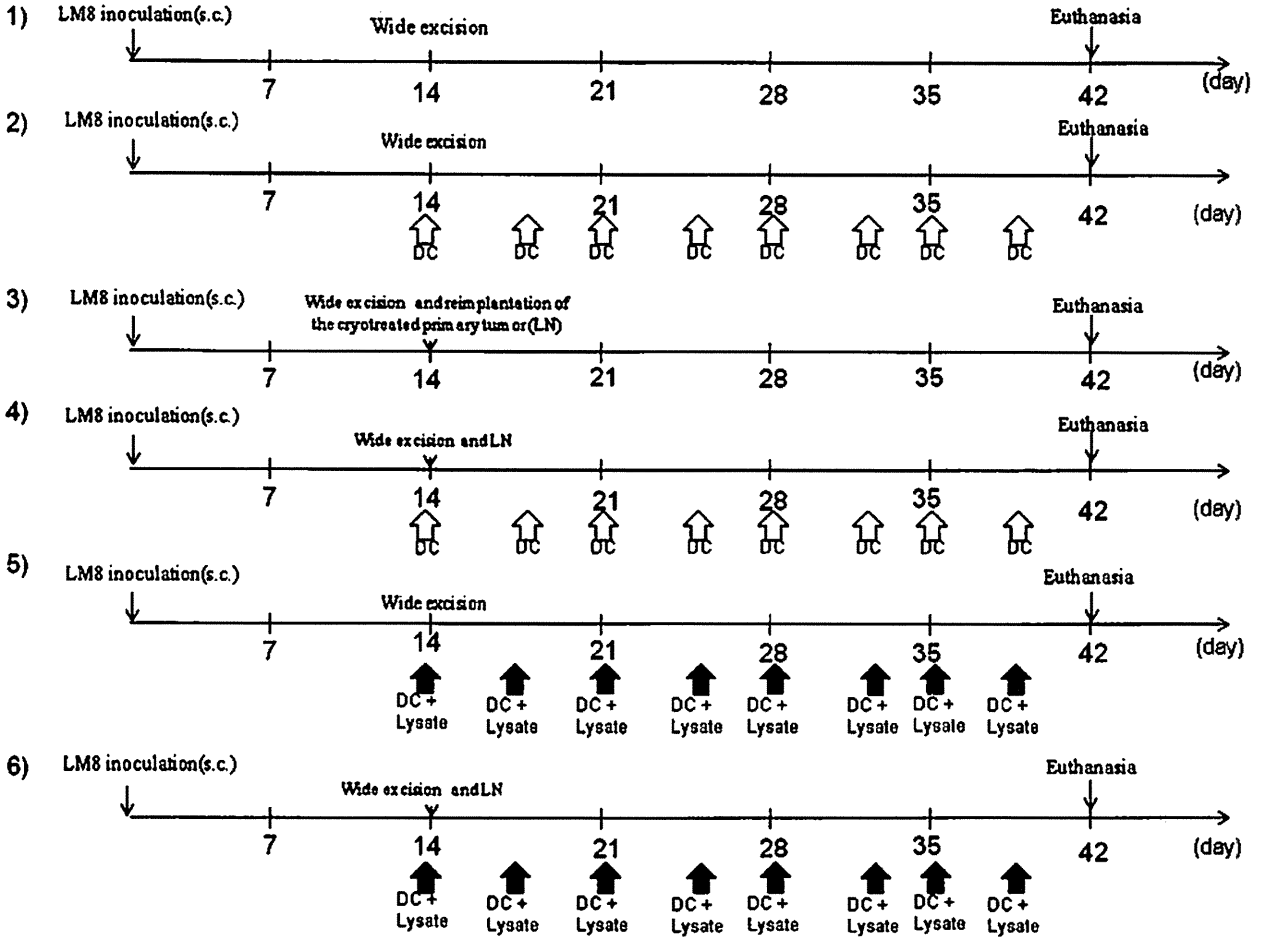


Fig. 1 A diagram of the experimental protocol and treatment schedule is shown. Two weeks after tumor inoculation, tumors were treated by one of the following methods: (1) excision only (n = 15); (2) DCs without reimplantation of the cryotreated primary tumor (n = 15); (3) reimplantation of the cryotreated primary tumor (n = 15); (4) DCs pulsed with cryotreated tumor lysates and reimplantation of the cryotreated primary tumor (n = 15); (5) DCs pulsed with cryotreated tumor lysates without reimplantation of the cryotreated primary tumor (n = 15); or (6) DCs pulsed with cryotreated tumor and reimplantation of the cryotreated primary tumor (LN) (n = 15). The mice were euthanized and evaluated 6 weeks after tumor inoculation. sc = subcutaneous.

gluteal region of the mice. All animals had macroscopically and microscopically confirmed lung metastases within 4 weeks [1].

C3H mice were purchased from Sankyo Labo Inc (Toyama, Japan) and housed in a specific pathogen-free animal facility in our laboratory. We were not able to accurately determine the survival time of each group because the guidelines for animal experiments concerning pain required euthanasia in distressed animals.

Liquid nitrogen (−196°C) was used for cryotreatment. Tumor tissue was collected on gauze and soaked in liquid nitrogen for 20 minutes for en bloc tumor tissue freezing. The tumor was prethawed at room temperature (20°C) for 15 minutes and then thawed in distilled water (20°C) for 15 minutes. The liquid nitrogen-treated tumor tissue was transplanted subcutaneously in the left gluteal region of the same mouse.

Because the mice were genetically identical, the structure of the major histocompatibility complex (MHC) Class I molecules was such that the T cells would be able to recognize the MHC Class I with antigens on the antigen-presenting cells (APCs) [17, 27]. Bone marrow-derived DCs were generated as described by Lutz and Rössner [28] with minor modifications. Briefly, erythrocyte-depleted mouse bone marrow cells obtained from flushed marrow cavities (1×10^6 cells/mL) were cultured in complete medium with 20 ng/mL recombinant mouse GMCSF (PeproTech EC Ltd, London, UK) in 10-cm tissue culture dishes at 37°C in an atmosphere containing 50 mL CO_2 per L. On Days 3 and 6, half of the medium was added to the

same volume of fresh complete medium and used to replenish the original plates. The freeze-thawed tumor lysate was added to the DC cultures on Day 6 at a ratio of five DC equivalents to one tumor cell (ie, 5:1) and incubated at 37°C in an atmosphere containing 50 mL CO_2 per L. After 24 hours of incubation, nonadherent cells including DCs were harvested by gentle pipetting.

For fluorescence activated cell sorting (FACS) analysis, DCs were counted with a FACSCalibur™ Flow Cytometer (Becton-Dickinson, San Jose, CA) and stained with fluorochrome-conjugated antibodies (BD Pharmingen, Tokyo, Japan) for the following markers: cluster of differentiation (CD)11c, CD80, CD86, I-Ad, and CD40. CD11c was used as a marker for all DCs regardless of the degree of maturation, whereas CD80, CD86, I-Ad, and CD40 are markers for DCs. Data analysis was performed with CELLQuest™ software (Becton-Dickinson). The corresponding labeled isotype antibodies served as controls. DCs used for vaccination were washed twice, enumerated, and resuspended in PBS at 1×10^6/mL.

We inoculated LM8 cells (5×10^6) in a mouse to make the tumor lysate. After 4 weeks, we resected the tumor mass and soaked the entire tumor in liquid nitrogen to kill the tumor cells. We mixed cryonecrotic tissue with DCs at Culture Day 6, after the tumor was defrosted, and the homogenate was prepared using PBS. The homogenate was passed through a 0.2-μm filter to remove bacteria and tissues and mixed with the DCs for 24 hours.

After intraperitoneal injection of 5 mL sodium pentobarbital (Somnopentyl®; Kyontsu Seiyaku, Tokyo, Japan), mice were euthanized by cervical dislocation and their blood was collected. Murine interferon (IFN)-γ and interleukin (IL)-4 release were measured by ELISA using Quantikine® (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol using an Easy Reader EAR340 microtest plate reader (SLT-Labinstruments, Salzburg, Austria).

We estimated the area of the pulmonary metastatic lesion on 50 serial histologic sections of each lung by manually drawing orthogonal lines delimiting the edges of the pulmonary metastatic lesion and selected the widest part of the specimen. The area was determined by multiplying the maximum orthogonal dimensions using ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). We compared the mean areas between the six groups.

For immunohistochemistry, lung specimens were fixed in 20% formalin and embedded in paraffin. For each case, we examined all the blocks of lung tissues of formalin-fixed, paraffin-embedded tumor tissue. All specimens were decalcified, although we found the decalcification step did not influence the immunohistochemistry for any of the stains. Five sections for each mouse were cut 4-μm thick. Each section was cut at the maximum diameter. CD8(+) T lymphocytes and natural killer (NK) cells in the pulmonary metastatic lesion were quantified by measuring the immunohistochemistry-positive cells per unit area in each group. Rehydrated tissue sections were incubated with rat monoclonal antibody raised against CD8(+) T lymphocytes of mouse origin (Santa Cruz Biotechnology, Santa Cruz, CA) and rat monoclonal antibody raised against NK cells of mouse origin (Abcam Plc, Cambridge, UK). The two antibodies were diluted 1:50 with PBS. Color reactions were performed at room temperature for 15 minutes and cover slips were mounted with glycerol and gelatin.

We determined differences in serum IFN-γ, serum IL-4, pulmonary metastatic area, and number of CD8(+) lymphocytes and NK cells in the metastatic area among the six groups using a nonrepeated-measures ANOVA and the Scheffe test. All analyses were conducted with SPSS® 11.0 software (SPSS Japan Inc, Tokyo, Japan).

Results

We activated antitumor immunity by combining DCs exposed to lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor. On Culture Day 7, the ratio of mature DCs to immature DCs was increased compared with the ratio at Culture Day 6 (Fig. 2; immature DCs, upper left; mature DCs, upper right). Moreover, this increase was more apparent in groups incubated with tumor lysate. Serum IFN-γ levels were greater ($p < 0.0001$) in the mice that received DCs combined with reimplantation of the cryotreated primary tumor (119.0 ± 7.61 pg/mL) than in the cryotreated primary tumor alone group (37.33 ± 2.58 pg/mL). Moreover, the group that received tumor lysate-exposed DCs combined with reimplantation of the cryotreated primary tumor (157.33 ± 14 pg/mL) had a greater ($p < 0.0001$) IFN-γ level than the group that received only tumor lysate-exposed DCs without reimplantation of the cryotreated primary tumor (120.27 ± 11.29 pg/mL) (Fig. 3). Serum IL-4 was lower ($p < 0.0001$) in the mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group (13.33 ± 9.75 pg/mL) than in the excision-only group (45.06 ± 5.71 pg/mL) (Fig. 4).

The enhanced immune response by T lymphocytes reduced metastatic lesions. Reduction of the metastatic area was greater ($p < 0.0001$) in the group that received DCs without reimplantation of the cryotreated primary tumor (15.99 ± 3.93 mm^2) than in the excision-only group (24.12 ± 3.60 mm^2). The reduction of the metastatic area was greater ($p < 0.0001$) in the DCs combined with reimplantation of the cryotreated primary tumor group (5.39 ± 1.49 mm^2) than in the reimplantation of

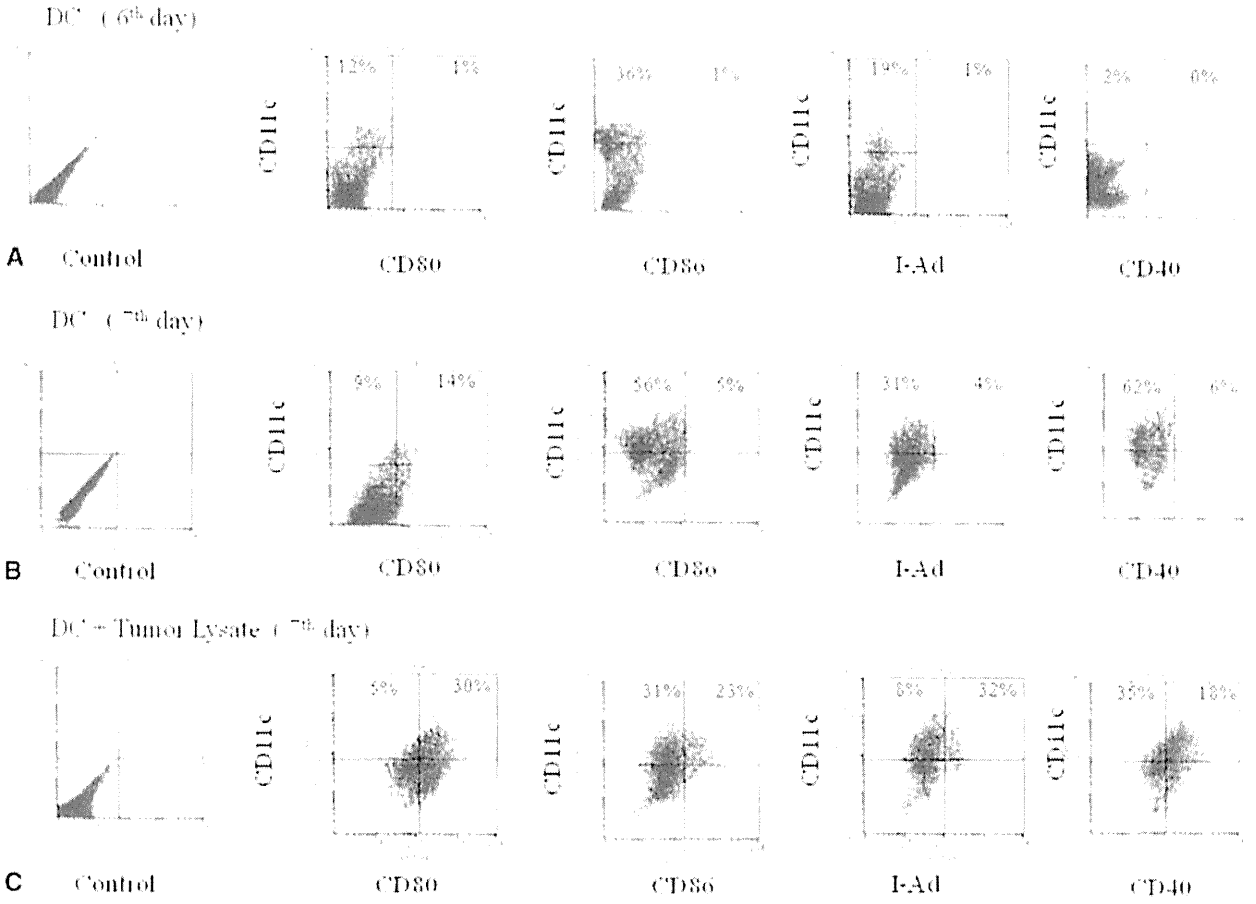
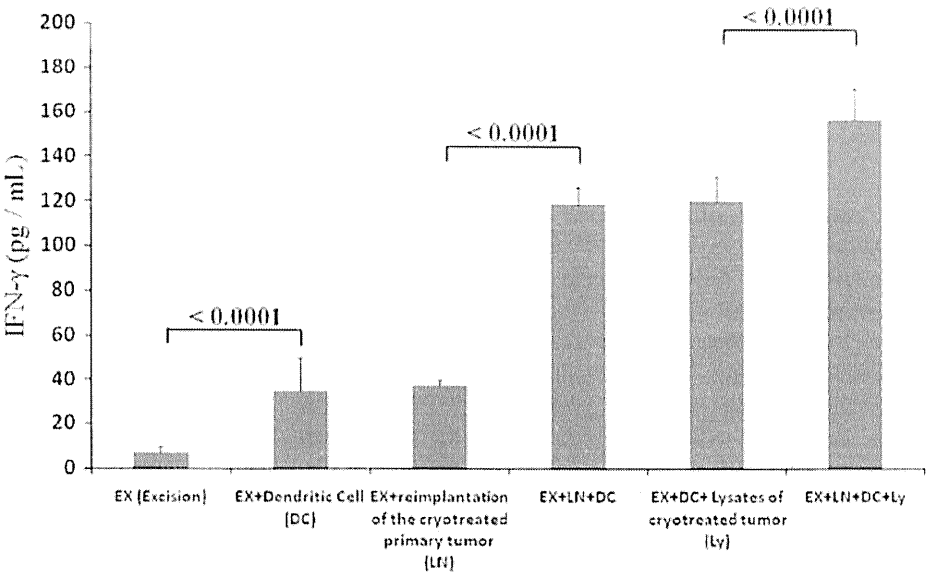


Fig. 2A–C DC activation status was examined using flow cytometry. DCs at Culture Day 7 (Group B) were more mature than DCs at Culture Day 6 (Group A). On Culture Day 7, DC maturity was greatest in the groups receiving lysate-primed DCs (Group C) than in those not receiving lysate-primed DCs (Group B).

Fig. 3 A graph of the serum IFN-γ levels in the six treatment groups is shown. The samples were collected 28 days after the reimplantation surgery and/or DC adoptive transfer. Mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a highest IFN-γ level. Error bars represent SD.



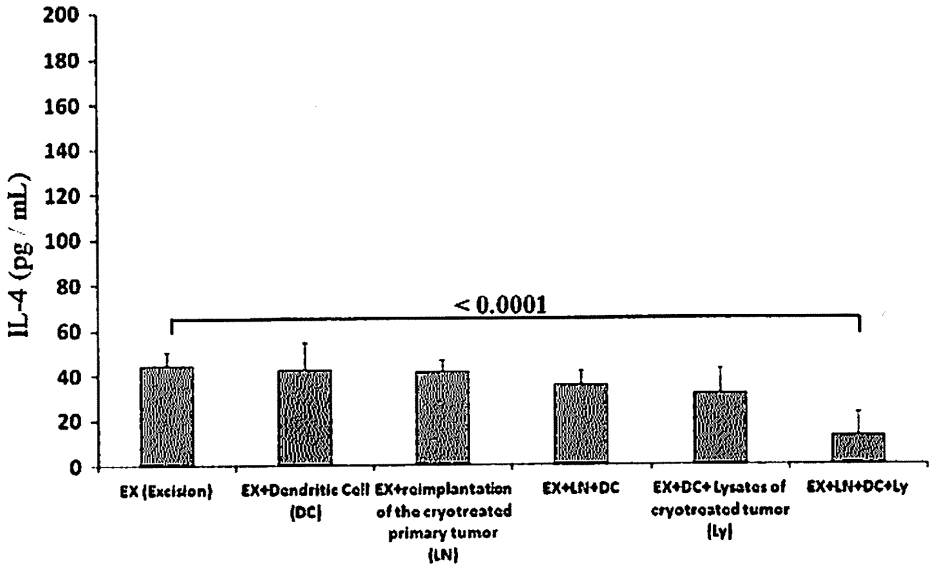


Fig. 4 A graph of the serum IL-4 in the six treatment groups is shown. Sera were collected 28 days after the reimplantation surgery and/or DC adoptive transfer. DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a lower level than any of the other groups. Error bars represent SD.

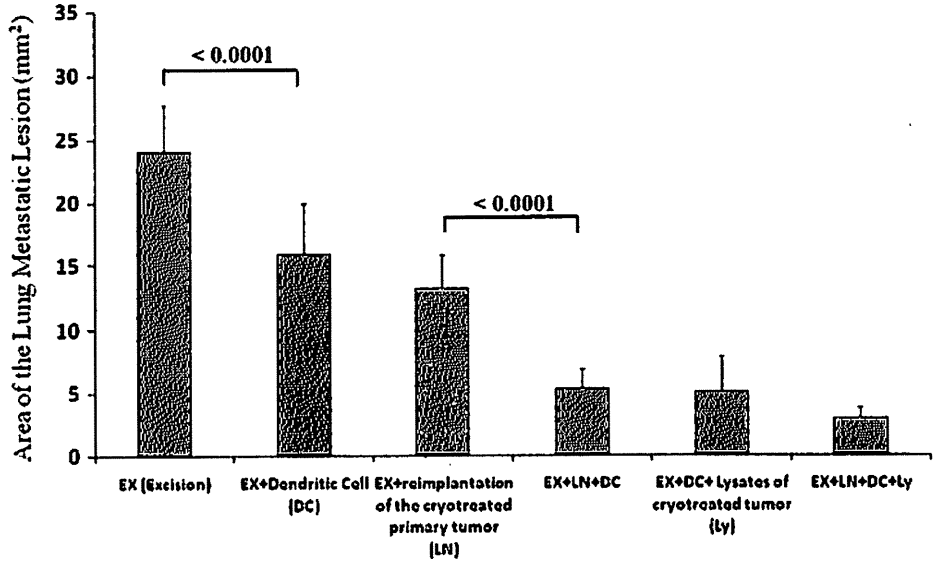


Fig. 5 Reduction of the metastatic area in the six treatment groups is shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. Error bars represent SD.

the cryotreated primary tumor alone group (13.22 ± 2.59 mm^2) (Fig. 5). CD8(+) T lymphocytes gathered in the pulmonary metastatic area in DC-treated groups, however, NK cells were not recruited to the metastatic area in the DC-treated groups compared with the nonDC-treated groups (Fig. 6). The number of CD8(+) T lymphocytes per unit area was greater ($p < 0.0001$) in the DCs combined with reimplantation of the cryotreated primary tumor group (8.33 ± 2.57 cells/mm^2) than in the reimplantation of the cryotreated primary tumor alone group (2.44 ± 0.53 cells/mm^2). Mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor (12.79 ± 2.14 cells/mm^2) showed higher ($p < 0.0001$) levels than the group that received DCs exposed to the lysates of cryotreated tumor without reimplantation of the cryotreated primary tumor (8.71 ± 2.39 cells/mm^2) (Fig. 7). The number of NK cells per unit area was greater ($p < 0.0001$) in the group that received DCs exposed to the lysates of cryotreated tumor without reimplantation of the cryotreated primary tumor (3.90 ± 2.17 cells/mm^2) than in the excision-only group (1.20 ± 0.30 cells/mm^2) (Fig. 8). The CD8(+)T lymphocyte, CD4(+) T lymphocyte, and DC infiltrations in reimplanted tumors were similar to those seen with pulmonary metastases (data not shown).

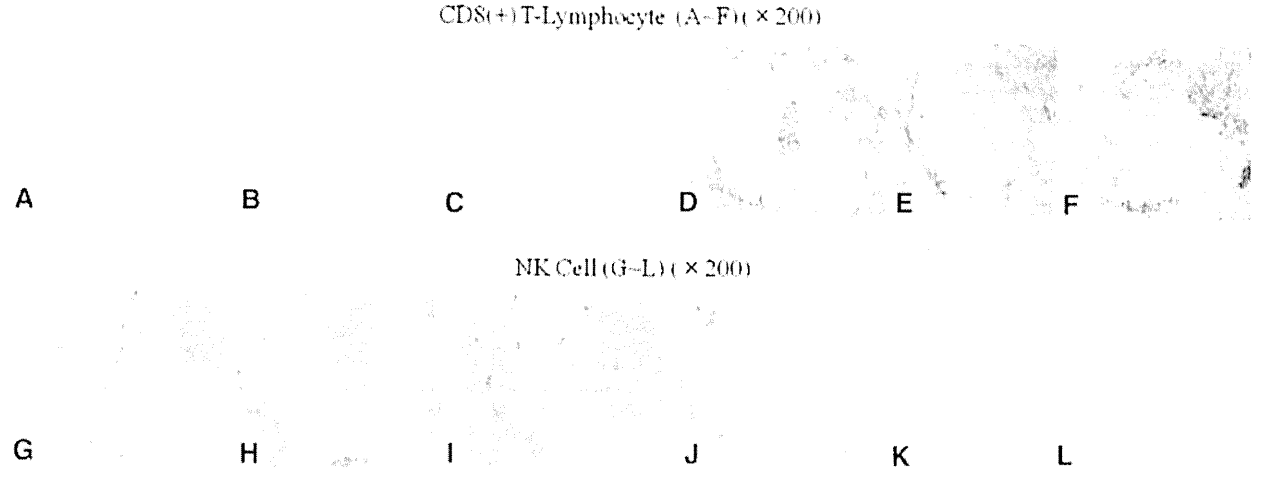


Fig. 6A–L To evaluate CD8(+) T lymphocytes and NK cells in pulmonary metastasis, immunostaining was performed: (**A**) CD8(+) T lymphocytes in Group 1, (**B**) CD8(+) T lymphocytes in Group 2, (**C**) CD8(+) T lymphocytes in Group 3, (**D**) CD8(+) T lymphocytes in Group 4, (**E**) CD8(+) T lymphocytes in Group 5, (**F**) CD8(+) T lymphocytes in Group 6, (**G**) NK cells in Group 1, (**H**) NK cells in Group 2, (**I**) NK cells in Group 3, (**J**) NK cells in Group 4, (**K**) NK cells in Group 5, and (**L**) NK cells in Group 6. CD8(+) T lymphocytes gathered in Groups D,E, and F. However, they did not gather in Groups A, B, and C. However, NK cells were recruited only in Groups A, B, and C. (Original magnification, ×200).

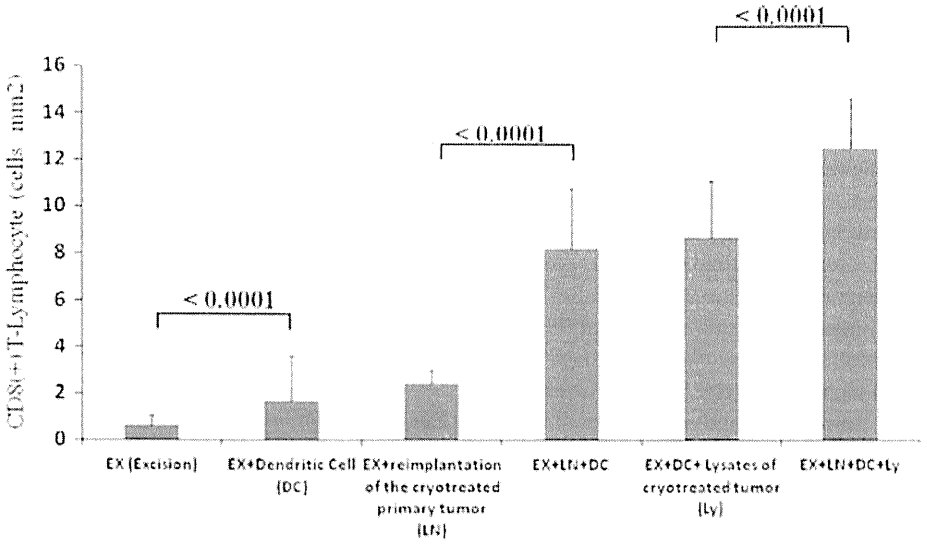


Fig. 7 The numbers of CD8(+) T lymphocytes per unit area in the six treatment groups are shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a higher level than any other groups. Error bars represent SD.

Discussion

Various immunotherapies for osteosarcoma have been tried. As standard treatments for osteosarcoma are ineffectual for many patients, new treatments need to be developed. In the 1970s, immunotherapy for osteosarcoma was reported by Southam et al. [42], Neff and Enneking [34], and Campbell et al. [5]. In the 1980s, new methods such as the use of interferons and Bacille de Calmette et Guérin were reported [22, 24, 36]. Another approach used antiidiotypic antibodies using T cells and liposome encapsulation [18, 51, 52]. Current methods of immunotherapy for osteosarcoma include peptide therapy or gene transfer therapy combined with hyperthermia therapy [10, 15, 21, 25, 33]. We asked whether (1) antitumor immunity could be achieved through activation of DCs combined with reimplantation of the cryotreated primary tumor and (2) if metastatic lesions would be prevented owing to enhanced T lymphocyte involvement.

We acknowledge limitations in this study. First, we used mice with an identical genetic makeup. The structure of the MHC Class I molecules was similar and the T cells could recognize the MHC Class I. However, we needed to use DCs from a different (albeit genetically identical) mouse to

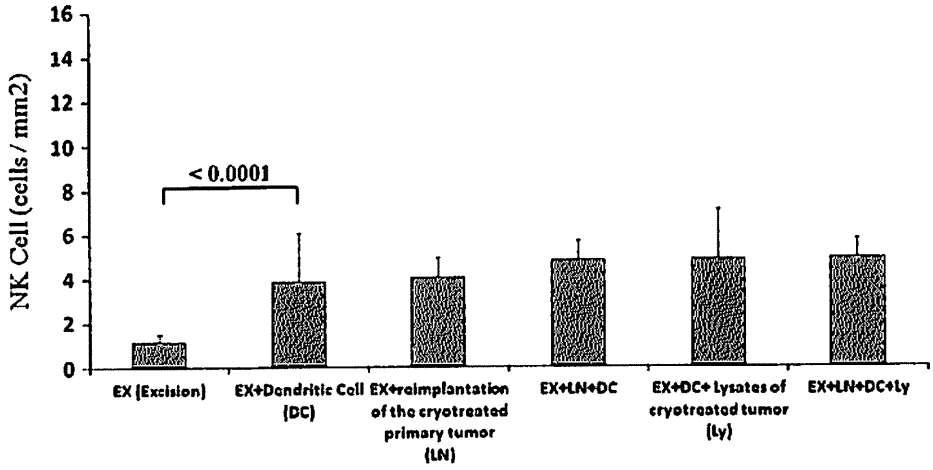


Fig. 8 The numbers of NK cells per unit area in the six treatment groups are shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. Error bars represent SD.

accomplish our adoptive transfer experiments. We minimized the potential for an immune response to nonself antigens by using genetically identical tumor tissue and mice. It would be necessary to use DCs derived from the same individual in clinical application, but this could not be achieved in our mouse model. In humans, however, monocytes are separated from the patient's own peripheral blood and DCs can be induced from these monocytes. Second, we could not completely replicate the clinical approach used in humans in our mouse model. In clinical cases frozen bone always is returned to the same site. However, it was impossible to replicate this in our experimental mouse model in which transplanted tumor cells were removed from the tibia and then returned to the same place after cryotreatment. In a preliminary experiment we attempted to do just that and these 20 mice could not move and died of starvation. We therefore used the contralateral gluteal region to check for local recurrence after tumor excision or recurrence from frozen tissue.

Antitumor immunity appeared to be activated through DCs combined with reimplantation of the cryotreated primary tumor or by exposing the transferred DC to lysates of cryotreated tumor. The use of lymphokine-activated killer (LAK) therapy has been used with other types of tumors [26]. However, T lymphocytes, which are the effectors, do not accumulate inside osteosarcoma tumors as expected. Autoclaving supplemented by DCs is thought to enhance the antitumor effect, but hyperthermia causes proteins to denature, and activation of the antitumor effect is often insufficient [37]. Several studies [12, 31, 41] report peptide vaccine therapy, but many patients apparently develop immunotolerance [45]. Thus, immunotherapy for malignant tumor achieved by these various methods has not been established definitively although investigations continue to try to overcome the major hurdles associated with immunotherapy (Table 1). We emphasize the immune response is activated by cryotreatment but not by heat-treated tissue. Our method differs from those described by others [7, 9, 10, 14]. In some regards DCs are believed to be the principal APCs for initiating immune responses in vivo [32]. In comparison with other traditional adjunct therapeutic options for cancer, such as radiation therapy and chemotherapy, immunotherapy provides a more targeted treatment to the cancer, with potentially fewer detrimental effects on noncancerous cells [30, 40]. DCs without sufficient cancer antigens may not have the ability to kill tumor cells and present the antigen to T lymphocytes by themselves. Our data suggest the antitumor effect in the group that received DCs without reimplantation of cryotreated primary tumor was almost the same as that in the reimplantation of cryotreated primary tumor alone group. The data further suggest the effects increased only when exposing the DCs to tumor lysates in the absence of cryonecrotic primary tumors. However, combining reimplantation of cryotreated primary tumor and DCs exposed to cryotreated tumor lysates produced synergistic effects. Using reimplantation of cryotreated primary tumor is more appropriate for clinical applications. We therefore believe an efficient immune response will be activated when DCs recognize tumor antigens appropriately. CD8(+) T cells act as an effector by the Th1 route, and this is promoted mainly by IFN-γ and IL-12 [38]. However, IL-4 [21], IL-6, and IL-10 strengthen humoral immunity. Levels of IFN-γ, IL-2, and IL-12 generally increase when cell-mediated immunity is activated, and IL-4, IL-6, and IL-10 increase when humoral immunity is activated. These cytokines act in opposition to maintain an immune balance.

Our data suggest enhanced T lymphocyte recruitment and function reduce metastatic lesions in a murine osteosarcoma model. Immunoreactivity increased slightly in mice that received DCs exposed to lysates of cryotreated tumor combined with reimplantation of the cryotreated primary tumor. NK cells attack the tumor independently of APCs. NK cells attack cells that downregulate MHC Class

Table 1. Immunotherapeutic trials of malignant tumors

Tumor	Immune intervention	Route	Immunologic response	Comments	References
Osteosarcoma	BCG	SC	NC	No consistent clinical effect	[22, 24]
Osteosarcoma	Interferon α	SC, IV	PR–NC	Osteosarcoma-associated antigens have potential for targeted immunotherapy	[36]
Unknown	LAK	IV	NC	T lymphocytes were unable to penetrate the tumor	[26]
Osteosarcoma	Antiidiotypic antibodies	IV	NC	It may be possible to circumvent this heterogeneity by activation of tissue macrophages to the tumoricidal state	[18, 51, 52]
Breast cancer, osteosarcoma	Peptide therapy combined with hyperthermia therapy	SC, IV	NC	It may be a potential agent for use in immunotherapy	[15, 20]
Osteosarcoma	Gene transfer therapy combined with hyperthermia therapy	IV	NC	IL-23 seems to be a less effective immunotherapeutic for adjuvant treatment of osteosarcomas	[25, 33]
Unknown	Peptide vaccine therapy	SC	NC–PD	Many patients have peptide-induced tolerance develop	[45]
Osteosarcoma	Cryoimmunology and DCs	SC	PR	Combining cryotreatment with DCs resulted in enhanced antitumor effects	Our data

BCG = Bacille de Calmette et Guérin; SC = subcutaneous; NC = no change; IV = intravenous; PR = partial response; LAK = lymphokine-activated killer; IL = interleukin; PD = progressive disease; DCs = dendritic cells.

I expression or have a stressed appearance [44]. We observed a reduced tumor burden in the groups that received transplanted DCs, which correlated with recruitment of CD8 lymphocytes to the tumor site as observed with immunohistochemistry.

Returning the frozen bone after liquid nitrogen treatment to its original place can be readily used in the clinic. After the first cryotreatment, it is possible to perform the treatment again using cultured DCs if a patient's tumor cells have been preserved. This approach therefore still can be used even after other methods (such as chemotherapy, radiation therapy, or surgery) no longer are reasonable. Combining DCs pulsed with lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor enhanced antitumor effects. We believe the approach may be a useful alternative for patients with osteosarcoma when other treatment options including chemotherapy, radiotherapy, and surgical treatment have been ineffective.

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Therapeutics, Targets, and Chemical Biology

Cancer Research

Antitumor Effect after Radiofrequency Ablation of Murine Hepatoma Is Augmented by an Active Variant of CC Chemokine Ligand 3/Macrophage Inflammatory Protein-1α

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Abstract

Several chemokines are used for immunotherapy against cancers because they can attract immune cells such as dendritic and cytotoxic T cells to augment immune responses. Radiofrequency ablation (RFA) is used to locally eliminate cancers such as hepatocellular carcinoma (HCC), renal cell carcinoma, and lung cancer. Because HCC often recurs even after an eradicative treatment with RFA, additional immunotherapy is necessary. We treated tumor-bearing mice by administering ECI301, an active variant of CC chemokine ligand 3, after RFA. Mice were injected s.c. with BNL 1ME A.7R.1, a murine hepatoma cell line, in the bilateral flank. After the tumor became palpable, RFA was done on the tumor of one flank with or without ECI301. RFA alone eliminated the treated ipsilateral tumors and retarded the growth of contralateral non–RFA-treated tumors accompanied by massive T-cell infiltration. Injection of ECI301 augmented RFA-induced antitumor effect against non–RFA-treated tumors when administered to wild-type or *CCR5*-deficient but not *CCR1*-deficient mice. ECI301 also increased CCR1-expressing $CD11c^+$ cells in peripheral blood and RFA-treated tumors after RFA. Deficiency of *CCR1* impairs accumulation of $CD11c^+$, $CD4^+$, and $CD8^+$ cells in RFA-treated tumors. Furthermore, in IFN-γ-enzyme-linked immunospot assay, ECI301 augmented tumor-specific responses after RFA whereas deficiency of *CCR1* abolished this augmentation. Thus, we proved that ECI301 further augments RFA-induced antitumor immune responses in a CCR1-dependent manner. *Cancer Res; 70(16); 6556–65. ©2010 AACR.*

Introduction

Chemokines are a class of candidate molecules for immunotherapy. Chemokines are presumed to play an essential role in the regulation of leukocyte trafficking and dendritic cell-T-cell interactions (1–4). In animal experiments, intratumoral use of chemokines, such as monocyte chemoattractant protein-1/CC chemokine ligand 2 (CCL2), macrophage inflammatory protein (MIP)-1α/CCL3, or MIP-3α/CCL20, succeeds in decreasing tumorigenesis accompanied by increase in the numbers of tumor-infiltrating dendritic, natural killer, or T cells (5–7). Thus, application of chemokines in immunotherapy is promising but needs further refinement before they can be used in clinical situations.

Radiofrequency ablation (RFA) is an eradicative treatment against cancers, such as hepatocellular carcinoma (HCC), renal cell carcinoma, and lung cancer. RFA of HCC can generate HCC-specific T cells in peripheral blood (8). Activation of dendritic cells in human peripheral blood is also observed after this treatment (9). Thus, RFA can induce immunogenic tumor cell death and subsequently tumor-specific immune responses (8–11). However, multicentric development of HCC in the cirrhotic liver frequently results in tumor recurrence even after the apparent curative treatment of HCC by RFA (12). These observations suggest that RFA-induced tumor-specific immune responses are often not sufficient to prevent tumor recurrence. Thus, additional treatment modalities are required to augment HCC-specific immune responses.

CCL3/MIP-1α can augment immune responses but problems arise because of its tendency to form large aggregates at high concentrations when administered systemically. Unlike human naïve CCL3, BB-10010 is generated by a single amino acid substitution of Asp26 to Ala and exhibits similar biological potencies, but rarely forms large aggregates (13). Based on its activity to mobilize bone marrow cells to peripheral blood, randomized clinical trials were performed to examine whether the combined administration of BB-10010 and chemotherapeutic agents can protect against chemotherapy-induced neutropenia. However, the myeloprotective effects of BB-10010 were not sufficient to warrant its use with chemotherapy (14). Concomitantly, several lines of evidence reveal that the administration of human recombinant CCL3

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can mobilize activated T-cell and dendritic cell precursors into circulation (15, 16).

ECI301, which has the same amino acid sequence as BB-10010, was generated using the fission yeast (*Schizosaccharomyces pombe*) expression system. ECI301 can augment irradiation-induced tumor regression when administered systemically to mice bearing multiple subcutaneous tumors (17). Of interest is the fact that the effects were observed in both unirradiated and irradiated tumors. Thus, systemic ECI301 treatment can augment irradiation-induced tumor-specific systemic immunity. These observations prompted us to investigate the effects of ECI301 on RFA-treated mice. Here, we show that ECI301 further augments RFA-induced antitumor immune responses in a CCR1-dependent manner.

Materials and Methods

Mice

Seven- to 9-week-old specific pathogen–free female BALB/c mice were purchased from Charles River Japan and designated as wild-type (WT) mice. BALB/c-*nu/nu* mice were purchased from CLEA Japan. *CCR1*-deficient (*CCR1*$^{-/-}$) mice were a gift from Dr. Philip M. Murphy (National Institute of Allergy and Infectious Disease, NIH, Bethesda, MD); *CCR5*-deficient (*CCR5*$^{-/-}$) mice were a gift from Dr. Kouji Matsushima (Department of Molecular Preventive Medicine, Tokyo University, Tokyo, Japan). All mice were backcrossed to BALB/c mice for 8 to 10 generations. All animal experiments were performed under specific pathogen–free conditions in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University (Japan).

Tumor cell line

A murine HCC cell line, BNL 1ME A.7R.1 (BNL), was purchased from the American Type Culture Collection in 1998 and kept at low passage throughout the study. The cells were screened for bacteria, fungus, and *Mycoplasma* contamination by direct culture method in 2006 before start of the study. The cells were cultured in DMEM (Sigma Chemical Co.) containing 10% fetal bovine serum (FBS), 0.1 mmol/L nonessential amino acids, 1 μmol/L sodium pyruvate, 2 mmol/L L-glutamine, 50 μg/mL streptomycin, and 100 units/mL penicillin (Life Technologies, Inc.).

Animal models

ECI301 was generated as previously described and provided by Effector Cell Institute, Inc. (17, 18). The left and right flanks of 7- to 9-week-old female WT, *CCR1*$^{-/-}$, *CCR5*$^{-/-}$, and *nu/nu* mice were injected s.c. with 5×10^5 BNL cells in 100 μL of PBS. Fourteen days later, when tumor size reached a diameter of 6 to 8 mm, tumors of one flank were treated using a radiofrequency generator (RITA 500PA, RITA Medical Systems) and needle as described below. On days 0, 2, and 4 after RFA, 20 μg of ECI301 in 100 μL of PBS were injected i.v. via the tail vein, whereas mice treated with RFA alone were injected with 100 μL of PBS. Untreated tumor-bearing mice were used as controls. In another schedule, 2 μg of ECI301 in 100 μL of PBS were injected i.v. from day 0 to day 4 (5 consecutive days). The sizes of non–RFA-treated tumors on the contralateral flank were evaluated twice a week using calipers, and tumor volumes were calculated using the following formula: tumor volume (mm^3) = (longest diameter) × (shortest diameter)^2 / 2.

RFA-treated or non–RFA-treated tumors were excised at the indicated time intervals for immunohistochemical analysis and quantitative real-time reverse transcription-PCR (RT-PCR). Spleens and peripheral blood were removed from the mice at the indicated time intervals for flow cytometric analysis and enzyme-linked immunospot assay (ELISPOT).

Radiofrequency ablation

Mice were anesthetized by i.p. injection of Somnopentyl (Schering-Plough Animal Health) and carefully shaved in the tumor area. After placing the mice onto an aluminum plate attached with an electricity-conducting pad, an RFA needle of expandable electrode with maximum dimension of 20 mm (70SB 2 cm; RITA Medical Systems) was inserted into the middle of the tumors and expanded at 2 or 3 mm. RFA treatments were done using a radiofrequency generator at a power output of 25 W for 1.5 minutes and the temperature of the needle tips reached 70°C to 80°C.

Immunohistochemical analysis

The removed tumor tissues were embedded in Sakura Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek) as frozen tissues. Cryostat sections of the frozen tissues were fixed with 4% paraformaldehyde in PBS and stained with rat anti-mouse CD4 (BD Biosciences), rat anti-mouse CD8a (BD Biosciences), hamster anti-mouse CD11c (BD Biosciences), and rat anti-mouse F4/80 antibodies (Serotec) overnight at 4°C. The sections were then incubated with biotinylated rabbit anti-rat IgG (DakoCytomation) or biotinylated mouse anti-hamster IgG (BD Biosciences) for 1 hour at room temperature. The immune complexes were visualized using the Catalyzed Signal Amplification System (DakoCytomation) or the Vectastain Elite ABC and DAB substrate kits (Vector Laboratories) according to the manufacturer's instructions. As a negative control, rat IgG (Cosmo Bio) or hamster IgG (BD Biosciences) was used instead of specific primary antibodies. The numbers of positive cells in each animal were counted in 10 randomly selected fields at 400-fold magnification by an examiner without any prior knowledge of the experimental procedures.

Double-color immunofluorescence analysis

Tumor tissues were embedded in OCT compound as frozen tissues. After fixation with 4% paraformaldehyde/PBS, cryostat sections were stained with the combinations of anti-CD4 and goat anti-mouse CCR1 (Santa Cruz Biotechnology), anti-CD8a and anti-CCR1, anti-F4/80 and anti-CCR1, phycoerythrin (PE)-conjugated hamster anti-CD11c (BD Biosciences) and anti-CCR1, anti-F4/80 and goat anti-mouse CCL3 (R&D Systems), and anti-F4/80 and goat anti-mouse CCL4 antibodies (R&D). After extensive washing, AF488 donkey anti-rat IgG (Invitrogen) was used as a secondary antibody to detect $CD4^+$, $CD8a^+$, or $F4/80^+$ cells. Simultaneously,