Cryoimmunologic antitumor effects enhanced by dendritic cells in osteosarcoma

Cryoimmunologic Antitumor Effects Enhanced by Dendritic Cells in Osteosarcoma

Running title: Cryoimmunology in Osteosarcoma

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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.

This work was performed at the Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University, and the Department of Orthopaedic Surgery, Faculty of Medicine, Oita University.

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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.

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

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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 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]. Several reports suggest the approach histologically 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.

コメント **[A1]:** AU: Confirm that Ref. 48 is correct here. The study from 1999 is reference 46; reference 48 was published in 2005 Response required.

Author response:
Reference 46 is the paper describing the intentional marginal excision in conjunction with caffeine-potentiated chemotherapy, not frozen autograft. The reference 48 describing f rozen autograft treated by liquid nitrogen was published in 2005. (the frozen autograft procedure was actually started from 1999.) Therefore, [48] is correct to be cited here..

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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, ~~which is similar to ours~~, promotes inflammation and activation of dendritic cells (DCs) that initiate the specific antitumor effect [19]. This type of immunotherapy is reportedly effective for breast and prostate cancer [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).

Material and Methods

Using a reported method to induce osteosarcoma [1, 35], we hypodermically implanted 1 x 10^6 LM8 cells (a murine osteosarcoma cell line) into the subcutaneous gluteal region of 80 female C3H mice, 6 to 8 weeks old. All animals developed tumors. 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 subcutaneous contralateral ~~subcutaneous~~ gluteal region without reimplantation of the cryotreated primary

コメント **[RAB2]:** AU: Confirm or correct. This is what you said in your response but did not put in the text. ED

Author response:
We delete this part because it is confusing. This method obtained antitumor effects by combining reimplantation of frozen tumor tissue with OK-432. It is similar to our methods because we combining reimplantation of frozen tumor tissue with DCs (instead of OK-432).

コメント **[RAB3]:** AU: This reference is for injecting tumor cells in the "back space' (presumably the authors meant in the subcutaneous tissues of the back, but that is unclear and "back space" is not a standard anatomical term). What did you do? See below. ED
The subcutaneous gluteal region is suitable expression.

コメント **[RAB4]:** AU: State where you implanted the tumor. Subcutaneous gluteal region? Flank? Elsewhere? Clarify in text. Response required on page proofs. ED

We implanted tumor cells in the subcutaneous gluteal region. We deleted ``flank``.

コメント **[RAB5]:** AU: Confirm or correct. Was this in the subcutaneous tissue or in muscle? ED

Author response: We injected tumor cells in into the subcutaneous contralateral gluteal region. We deleted ``flank``.

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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 subcutaneous 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 subcutaneous 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 subcutaneous 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 subcutaneous 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 ~~a mouse~~ another mice, and then the tumor was treated with liquid nitrogen to create the lysates. ~~Immunologic effectiveness would not change even when the transplants were returned to the contralateral flank.~~ We presumed a systemic immune response would be induced by injecting DCs around the frozen tumor tissue. We performed microscopy to determine whether metastasis had occurred in the lungs 2 weeks after the tumor inoculation. We confirmed the presence of pulmonary metastases in 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 cite 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

コメント **[RAB6]:** AU: Only one mouse? Why are you creating these lysates?

It dosen`t mean one mouse. It is just a explanation that we made tumor lysate from another mice to expose DCs in group (5) and (6). DCs were sensitized by tumor lysates.

コメント **[RAB7]:** AU: Confirm or correct. I reworded because your statement was unclear. ED

Author response:
It is just a explanation that we made tumor lysate from another mice to expose DCs in group (5) and (6).

コメント **[RAB8]:** AU: Unclear. Is this a statement of fact from the literature? If so, provide the citation. If not, why wouldn't the effectiveness change? Clarify in text. ED

Author response:
We deleted this sentence because it doesn't have the scientific evidence.

コメント **[RAB9]:** AU: What 20 mice? You have 80 or 90 total in each of six groups (15 each). They can't be the 20 treated with "tibial tumor-bearing bone" because those died of starvation, not metastases. Are these 20 additional animals? They are not on Figure 1. Clarify in text. ED

Author response:
In advance, we confirmed the presence of pulmonary metastases in another 20 mice in a preliminary experiment and they were not included in the six groups.

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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 gluteal region ~~flanks~~ 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 ~~flank~~ 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

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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 x 10^6/mL.

We inoculated LM8 cells (5 x 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

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protocol using an Easy Reader EAR340 microtest plate reader (SLT-Labinstruments, Salzburg, Austria).

We measured the area of the pulmonary metastatic lesion on the plane of the maximum ~~cut~~ dimension from 50 serial histological sections of each lung using ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). All areas were measured manually by drawing lines delimiting the edges of the pulmonary metastatic lesion. We compared the mean areas between the six groups.

コメント **[RAB10]:** AU: This is still unclear. Are these from histologic sections? Are they 3-D reconstructions from CT or from serial histological sections? Selected sections? If selected sections, how many did you examine? How did you determine the "plane of the maximum cut?" Do you mean the maximum dimensions? Clarify in text. ED

Author response:
We examined 50 serial histological sections of each lung and selected the plane of the maximum dimension. (We measured and selected the widest part of the specimen using Image-J)

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 coverslips 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).

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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 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

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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 was similar to that seen with pulmonary metastases (data not shown).

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 lymphocytes involvement.

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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 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 is always 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 ~~flank~~ to check for local recurrence after tumor excision or recurrence from frozen tissue.-

Antitumor immunity appeared 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. Hyperthermia, through autoclaving ~~or pasteurization,~~ with 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

コメント **[RAB11]:** AU: Confirm or correct. I had previously requested you mention this in Materials and Methods but I presumed these were part of your experiment of 80 (or 90) mice. It now appears this is not the case. Was this a preliminary experience? Clarify in text. ED

Author response:
We confirmed that 20 mice died of starvation after cryotreatment of tibia bearing tumor in a preliminary experiment and they were not included in the six groups.

コメント **[RAB12]:** AU: My understanding of pasteurization is the temperatures are sufficiently low so proteins do not denature? Clarify in text. ED

Author response:
You are right. 60 degrees centigrade keeps protein activities. Accordingly, I deleted `` pasteurization`` to avoid confusion.

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established definitively although investigations continued 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 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 reduces metastatic lesions in a murine osteosarcoma model. Immunoreactivity increased slightly in mice that received

コメント **[RAB13]:** AU: Your data does not show this prevents lesions, only reduces the area. ED
Yes, it just reduces the metastatic areas.

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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 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 can therefore still be used even after other methods, such as chemotherapy, radiation therapy, or surgery are no longer 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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Acknowledgments

We thank Katsuro Tomita, Akihiko Takeuchi, Shuichi Kaneko, and Yohei Marukawa for supervision in this study.

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AU: Please do not delete query boxes or remove line numbers; ensure you address each query in the query box.

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コメント **[B14]:** RB: This most likely is an Abstract as it is from conference proceedings. Is it ok to retain or do you want it deleted?

Please retain this reference if possible.

AU: Please do not delete query boxes or remove line numbers; ensure you address each query in the query box.

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AU: Please do not delete query boxes or remove line numbers; ensure you address each query in the query box.

Legends

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. s.c. = subcutaneous.

コメント **[RAB15]:** AU: Note the number of animals in each group. ED

Author response:
We added the number of animals in each group

コメント **[RAB16]:** AU: Confirm or correct. ED
It is correct.

Fig. 2 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).

コメント **[BP17]:** COMP: Ok to publish in color in print and online. Thanks.
It is OK.

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.

コメント **[BP18]:** AU: Is this sentence correct as rewritten? The legends should be written using complete sentences. Response required.

I confirmed this sentence is correct.

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 lower level than any other groups. Error bars represent SD.

AU: Please do not delete query boxes or remove line numbers; ensure you address each query in the query box.

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.

コメント **[BP19]:** COMP: Ok to publish this figure in color in print and online. Thanks.
It is OK.

Fig. 6

To evaluate CD8(+) T lymphocytes (Figures A, B, C, D, E and F) and NK cells (Figures G, H, I, J, K and L) in pulmonary metastasis, immunostaining was performed: ~~(A, G) Group 1, (B, H) Group 2, (C, I) Group 3, (D, J) Group 4, (E, K) Group 5, and (F, L) Group 6~~ 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, L; NK cells in Group 6. CD8(+) T lymphocytes gathered in group D,E and F. However, they did not gathered in group A, B, and C. On the other hand, NK cells were recruited only in group A, B and C. (Original magnification of each figure, x200).

コメント **[BP20]:** AU: You must have a separate legend for each part of the figure and each must make a separate point.

We modify and added sentence for each part

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.

コメント **[BP21]:** AU: Is this sentence correct as rewritten? Response required.

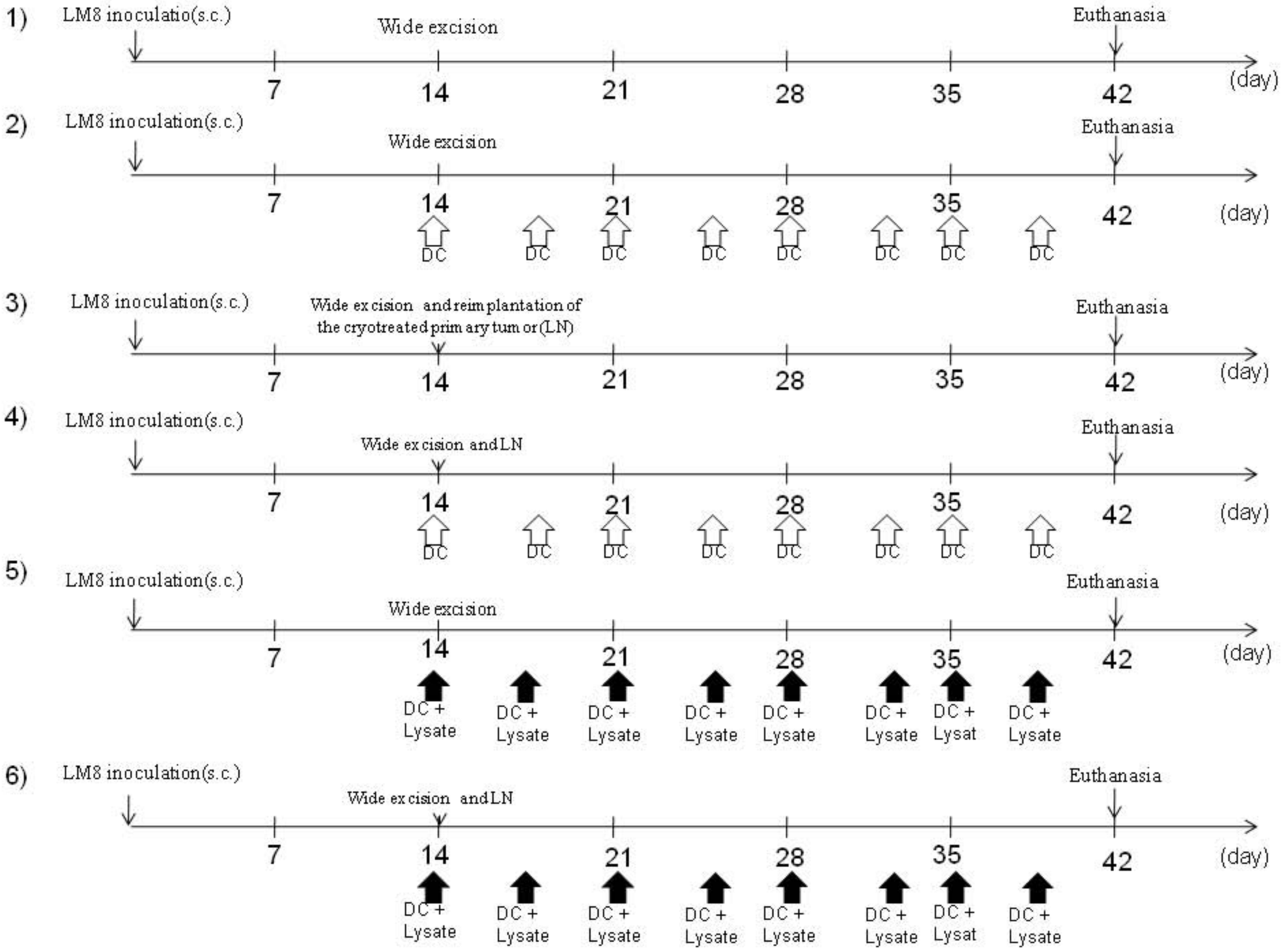
I confirmed this sentence is correct.

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.

コメント **[BP22]:** AU: Is this sentence correct as rewritten? Response required.

I confirmed this sentence is correct.

AU: Please do not delete query boxes or remove line numbers; ensure you address each query in the query box.


1)
LM8 inoculatio(s.c.)
Wide excision
Euthanasia
7
14
21
28
35
42
(day)
2)
LM8 inoculation(s.c.)
Wide excision
Euthanasia
7
14
21
28
35
42
(day)
DC
3)
LM8 inoculation(s.c.)
Wide excision and reimplantation of the cryotreated primary tumor(LN)
Euthanasia
7
14
21
28
35
42
(day)
4)
LM8 inoculation(s.c.)
Wide excision and LN
Euthanasia
7
14
21
28
35
42
(day)
DC
5)
LM8 inoculation(s.c.)
Wide excision
Euthanasia
7
14
21
28
35
42
(day)
DC + Lysate
DC + Lysate
DC + Lysate
DC + Lysate
DC + Lysate
DC + Lysate
DC + Lysat
DC + Lysate
6)
LM8 inoculation(s.c.)
Wide excision and LN
Euthanasia
7
14
21
28
35
42
(day)
DC + Lysate
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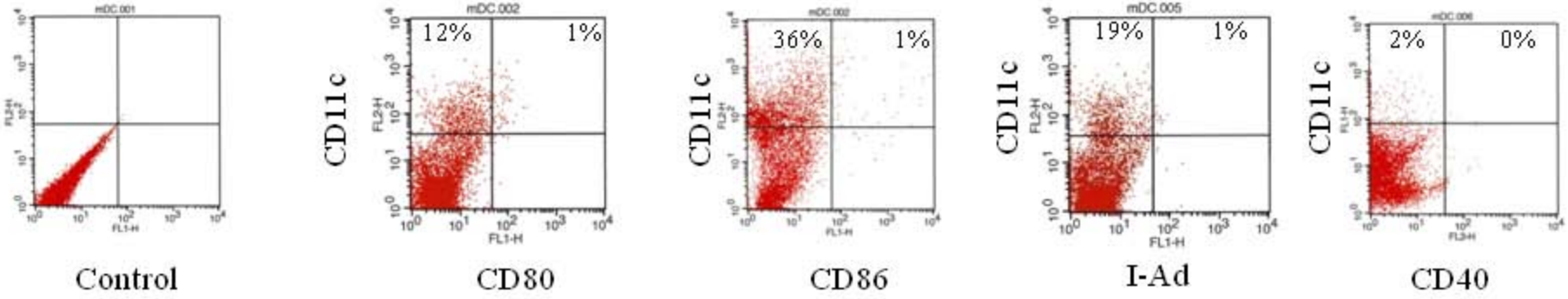
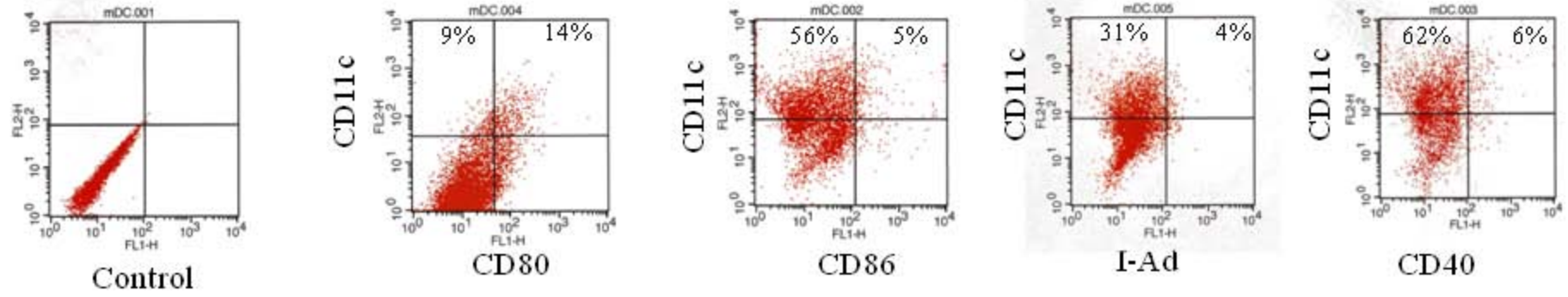
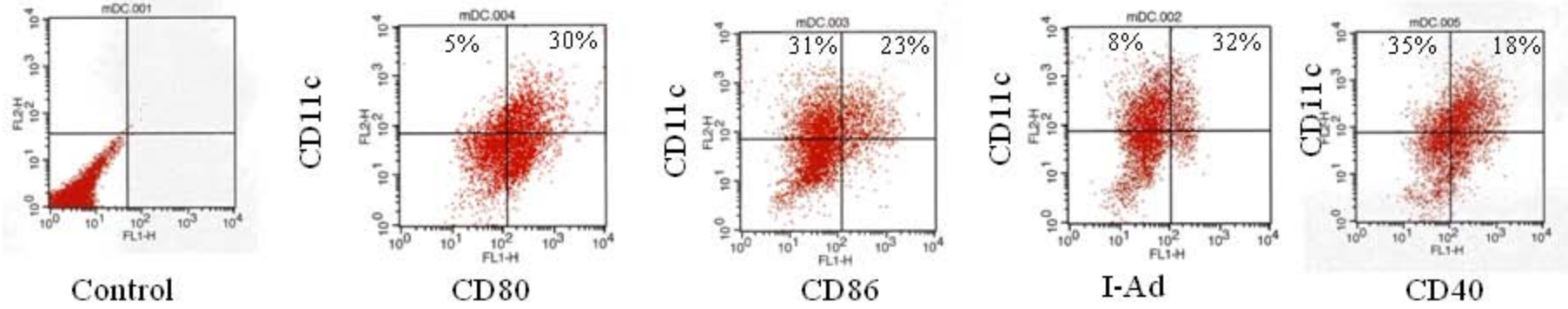
a . DC (6th day)

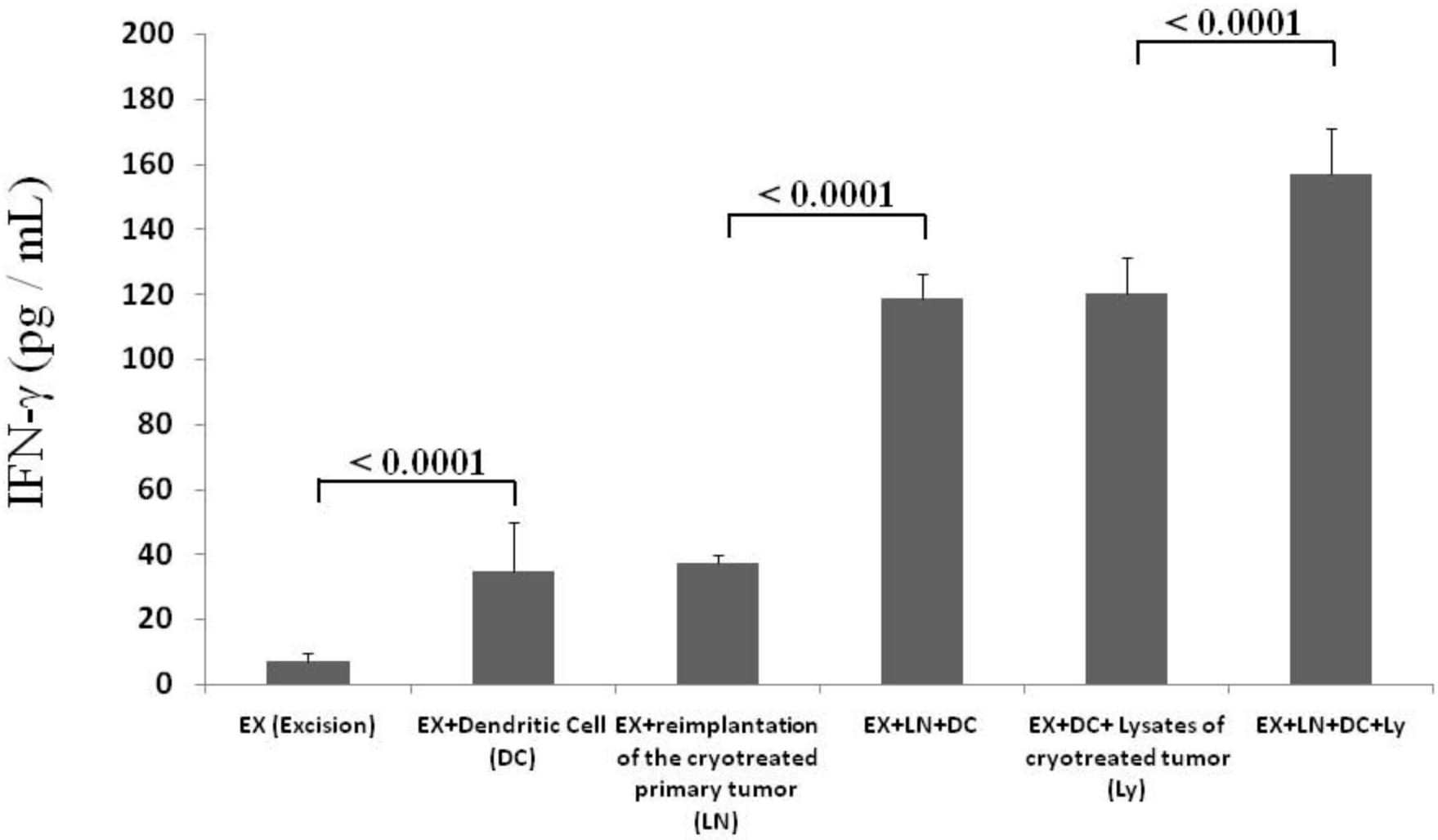


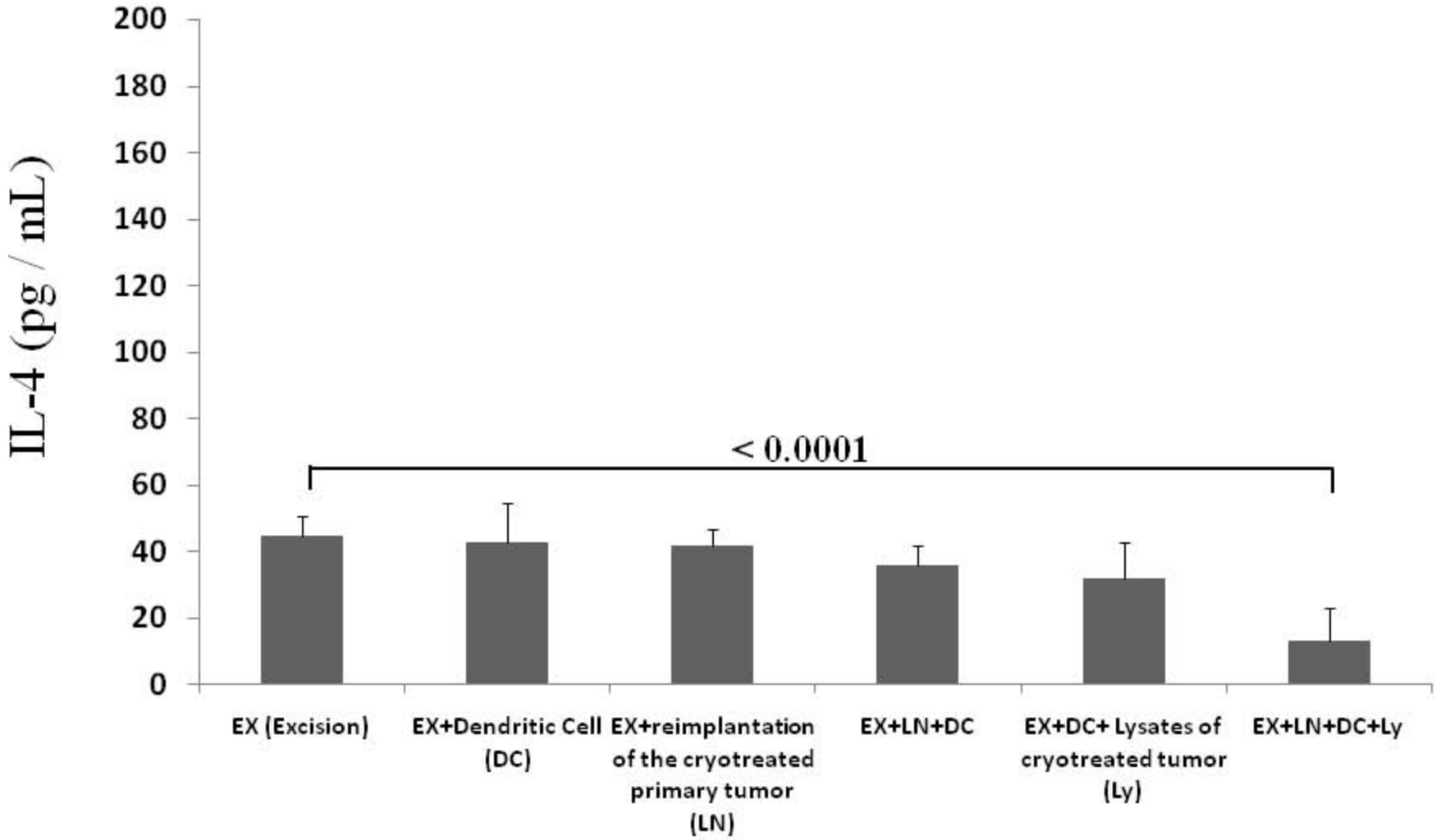
b . DC (7th day)

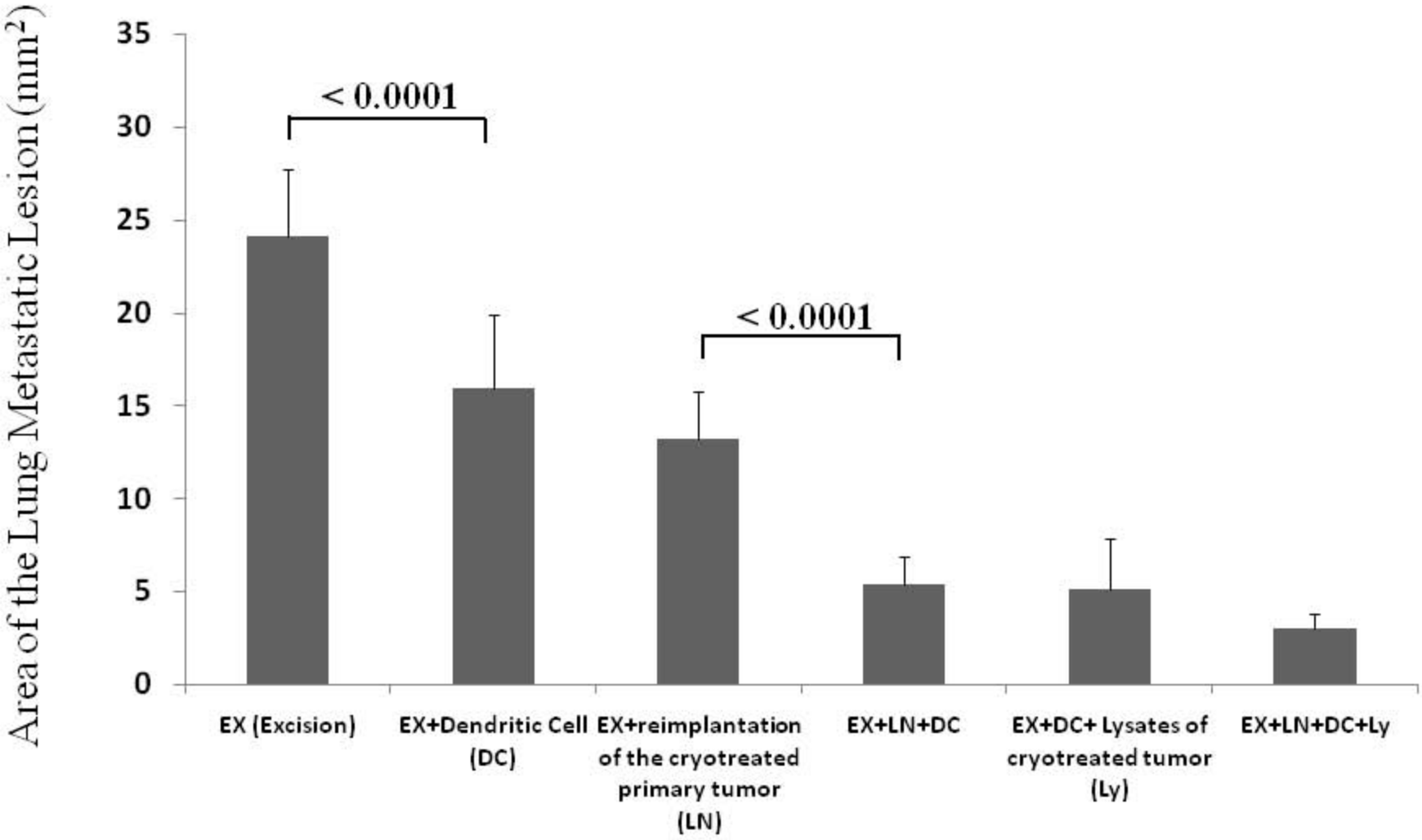


c . DC + Tumor Lysate (7th day)

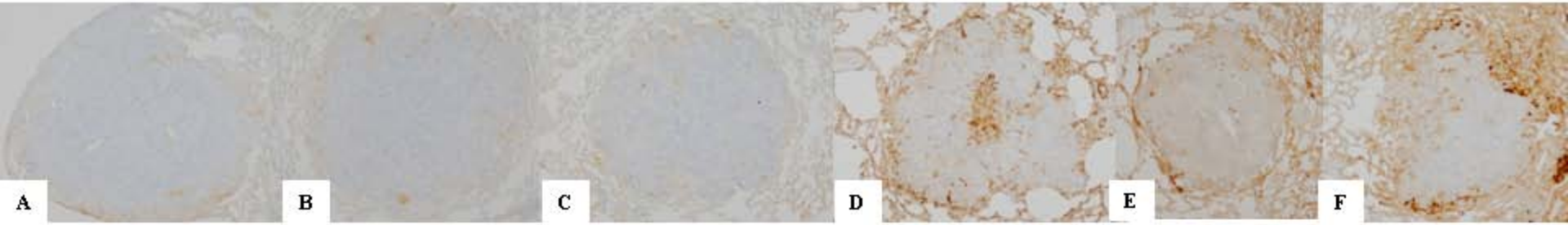



IFN-γ (pg / mL)
200
180
160
140
120
100
80
60
40
20
0
< 0.0001
< 0.0001
< 0.0001
EX (Excision)
EX+Dendritic Cell (DC)
EX+reimplantation of the cryotreated primary tumor (LN)
EX+LN+DC
EX+DC+ Lysates of cryotreated tumor (Ly)
EX+LN+DC+Ly

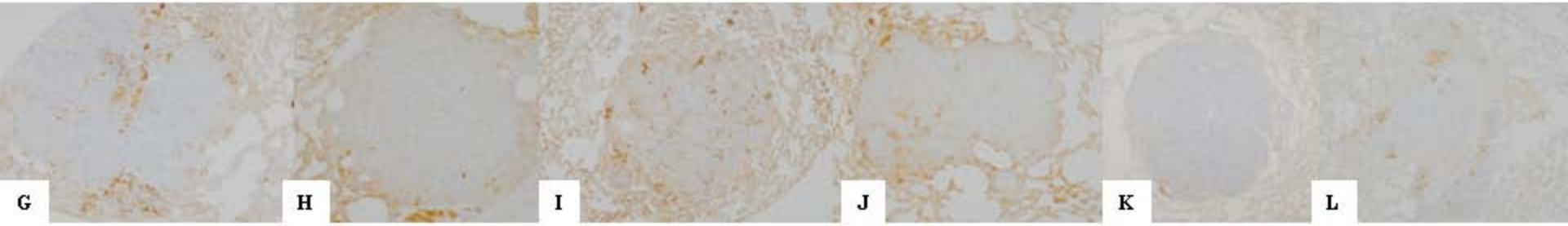

IL-4 (pg / mL)
200
180
160
140
120
100
80
60
40
20
0
< 0.0001
EX (Excision)
EX+Dendritic Cell (DC)
EX+reimplantation of the cryotreated primary tumor (LN)
EX+LN+DC
EX+DC+ Lysates of cryotreated tumor (Ly)
EX+LN+DC+Ly

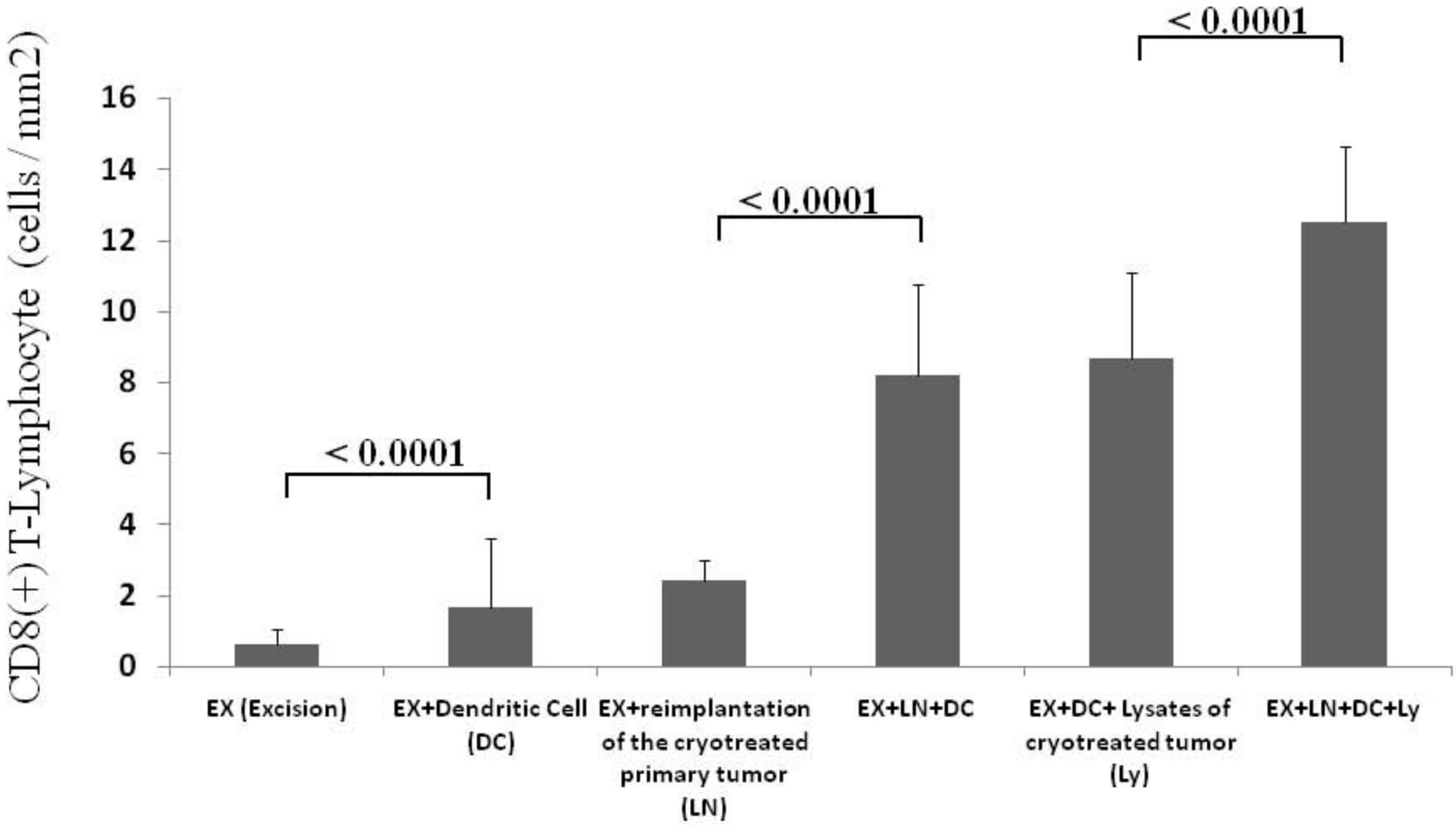

Area of the Lung Metastatic Lesion (mm²)
35
30
25
20
15
10
5
0
< 0.0001
< 0.0001
EX (Excision)
EX+Dendritic Cell (DC)
EX+reimplantation of the cryotreated primary tumor (LN)
EX+LN+DC
EX+DC+ Lysates of cryotreated tumor (Ly)
EX+LN+DC+Ly

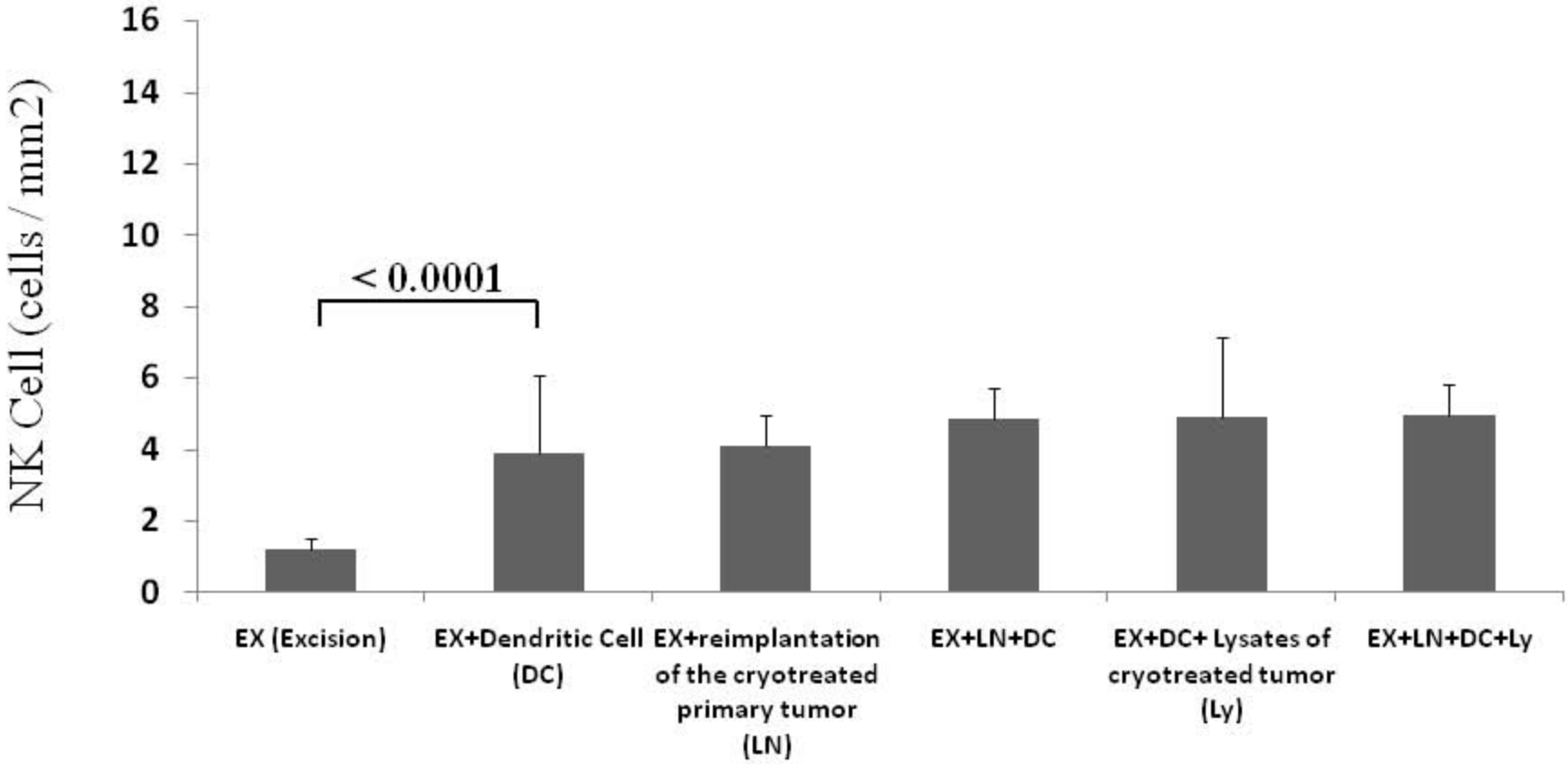
CD8(+) T-Lymphocyte (A~F) (× 200)



NK Cell (G~L) (× 200)




CD8(+) T-Lymphocyte (cells / mm2)
16
14
12
10
8
6
4
2
0
< 0.0001
< 0.0001
< 0.0001
EX (Excision)
EX+Dendritic Cell (DC)
EX+reimplantation of the cryotreated primary tumor (LN)
EX+LN+DC
EX+DC+ Lysates of cryotreated tumor (Ly)
EX+LN+DC+Ly


NK Cell (cells / mm2)
16
14
12
10
8
6
4
2
0
< 0.0001
EX (Excision)
EX+Dendritic Cell (DC)
EX+reimplantation of the cryotreated primary tumor (LN)
EX+LN+DC
EX+DC+ Lysates of cryotreated tumor (Ly)
EX+LN+DC+Ly