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Cryoimmunologic Antitumor Effects Enhanced by Dendritic Cells 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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1 **Abstract**

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

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

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

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

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

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

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24 Introduction

25 The standard treatment of osteosarcoma consists of preoperative chemotherapy, surgical
 26 tumor excision, and postoperative chemotherapy. Limb-saving surgery is feasible in most
 27 cases. Advances in osteosarcoma treatment have now achieved a 5-year survival rate of 60%
 28 to 90% for patients, and limb function after reconstruction continues to improve with time [3,
 29 16, 30, 46, 47, 49].

30 Tsuchiya et al. developed a new approach using frozen autografts [48] to improve
 31 reconstruction after osteosarcoma resection. The tumor is resected with an adequate margin,
 32 and the resected specimen is immersed in liquid nitrogen for 20 minutes to kill all tumor cells.
 33 After thawing, the specimen is returned to the original place with appropriate internal fixation
 34 to reconstruct the defect. Compared with heat-treated bones [8, 14], bone genetic proteins and
 35 native biomechanical structures are preserved after cryotreatment [53]. In one report limb
 36 function using the technique of Tsuchiya et al. was rated as excellent in 71.4% of patients,
 37 and good in 10.7%, as assessed by the functional evaluation system of Enneking [11]. Several
 38 reports suggest the approach histologically enhanced bone formation when compared
 39 histologically with pasteurized bone and irradiated bone [43,48]. Another advantage in
 40 reimplanting cryotreated tumor tissue is its effect on the immune system [50]: tumor tissue
 41 after cryoablation in situ provokes an immune reaction in patients with breast and prostate
 42 cancer [6, 8, 39]. Brewer et al. reported metastatic tumors sometimes disappear or shrink after
 43 in situ cryoablation of the primary tumor with liquid nitrogen [4]. The structure of tumor
 44 antigens is retained in frozen tumor, and leukocytes probably can recognize these antigens.
 45 Similar antitumor effects can be expected from our reconstructive procedure of reimplanting
 46 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 frozen 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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47 Nishida et al. observed an inadequate antitumor effect after reimplantation of frozen tumor
 48 tissue alone [35]. However, the antitumor effect was enhanced by promoting nonspecific
 49 immune activation by intraperitoneal injection of OK-432, a substance extracted from alpha-
 50 Streptococcus pyogenes. This approach, **which is similar to ours**, promotes inflammation and
 51 activation of dendritic cells (DCs) that initiate the specific antitumor effect [19]. This type of
 52 immunotherapy is reportedly effective for breast and prostate cancer [6, 8, 39]. Many groups
 53 have reported successful immunotherapy for osteosarcoma [5, 15, 18, 20, 22, 24, 25, 33, 34,
 54 36, 42, 51, 52]. However, the ability to control metastatic lesions and local recurrence does
 55 not appear to be superior to other adjuvant treatments [2, 7, 13, 23, 29].

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).

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

61 **Material and Methods**

62 Using a reported method to induce osteosarcoma [1, 35], we hypodermically implanted 1 x
 63 10⁶ LM8 cells (a murine osteosarcoma cell line) into **the subcutaneous gluteal region** of 80
 64 female C3H mice, 6 to 8 weeks old. All animals developed tumors. Two weeks after
 65 inoculation, we surgically excised the tumors and cryotreated them with liquid nitrogen. We
 66 established the following six groups (Fig. 1): (1) the tumor was excised with wide margins 14
 67 days after inoculation (n = 15); (2) the tumor was excised with wide margins 14 days after
 68 inoculation and bone marrow-derived DCs then were injected into **the subcutaneous**
 69 **contralateral subcutaneous gluteal** region without reimplantation of the cryotreated primary

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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70 tumor twice a week (n = 15); (3) the tumor was excised with wide margins 14 days after
 71 inoculation and reimplanted after cryotreatment with liquid nitrogen into the **subcutaneous**
 72 contralateral gluteal region to evaluate for local recurrence from frozen tumor tissue (n = 15);
 73 (4) the tumor was excised 14 days after inoculation and reimplanted after cryotreatment into
 74 the **subcutaneous** contralateral gluteal region to evaluate for local recurrence, and DCs then
 75 were injected twice a week into this secondary site (n = 15); (5) the tumor was excised with
 76 wide margins 14 days after inoculation and DCs exposed to cryotreated tumor lysates were
 77 injected twice a week into the **subcutaneous** contralateral gluteal region without
 78 reimplantation of the cryotreated primary tumor (n = 15); and (6) the tumor was excised with
 79 wide margins 14 days after inoculation and reimplanted after the treatment with liquid
 80 nitrogen into the **subcutaneous** contralateral gluteal region to evaluate for local recurrence
 81 (same as Group 3) with the addition of DCs exposed to cryotreated tumor lysates injected
 82 twice a week (n = 15). **We harvested tumor from a mouse another mice, and then the tumor**
 83 **was treated with liquid nitrogen to create the lysates. Immunologic effectiveness would not**
 84 **change even when the transplants were returned to the contralateral flank. We presumed a**
 85 systemic immune response would be induced by injecting DCs around the frozen tumor tissue.
 86 We performed microscopy to determine whether metastasis had occurred in the lungs 2 weeks
 87 after the tumor inoculation. **We confirmed the presence of pulmonary metastases in additional**
 88 **20 mice in a preliminary experiment in advance. We also confirmed that there were no viable**
 89 cells after cryotreatment using liquid nitrogen, in agreement with a previous study [35]. We
 90 observed no recurrence of the tumor at the primary cite of inoculation after excision. All
 91 experiments were performed under the guidelines for animal experiments as stipulated by the
 92 Kanazawa University Graduate School of Medical Science [37].
 93 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 doesn'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 rewored 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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94 Center (Saitama, Japan). The cells were maintained in complete medium consisting of RPMI
95 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 µg streptomycin per
96 mL, and 100 units penicillin per mL and were cultured at 37° C in 5% CO₂. To establish local
97 implantation of the tumor and subsequent lung metastasis, the LM8 cells (1 x 10⁶) were
98 suspended in 0.2 mL phosphate-buffered saline (PBS) and subcutaneously inoculated into the
99 right **gluteal region flank** of the mice. All animals had macroscopically and microscopically
100 confirmed lung metastases within 4 weeks [1].

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

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

110 Because the mice were genetically identical, the structure of the major histocompatibility
111 complex (MHC) Class I molecules was such that the T cells would be able to recognize the
112 MHC Class I with antigens on the antigen-presenting cells (APCs) [17, 27]. Bone marrow-
113 derived DCs were generated as described by Lutz and Rössner [28] with minor modifications.
114 Briefly, erythrocyte-depleted mouse bone marrow cells obtained from flushed marrow cavities
115 (1 x 10⁶ cells/mL) were cultured in complete medium with 20 ng/mL recombinant mouse
116 GM-CSF (PeproTech EC Ltd, London, UK) in 10-cm tissue culture dishes at 37° C in an

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117 atmosphere containing 50 mL CO₂ per L. On Days 3 and 6, half of the medium was added to
118 the same volume of fresh complete medium and used to replenish the original plates. The
119 freeze-thawed tumor lysate was added to the DC cultures on Day 6 at a ratio of five DC
120 equivalents to one tumor cell (ie, 5:1) and incubated at 37° C in an atmosphere containing 50
121 mL CO₂ per L. After 24 hours of incubation, nonadherent cells including DCs were harvested
122 by gentle pipetting.

123 For fluorescence activated cell sorting (FACS) analysis, DCs were counted with a
124 FACSCalibur™ Flow Cytometer (Becton Dickinson, San Jose, CA) and stained with
125 fluorochrome-conjugated antibodies (BD Pharmingen, Tokyo, Japan) for the following
126 markers: cluster of differentiation (CD)11c, CD80, CD86, I-Ad, and CD40. CD11c was used
127 as a marker for all DCs regardless of the degree of maturation, whereas CD80, CD86, I-Ad,
128 and CD40 are markers for DCs. Data analysis was performed with CELLQuest™ software
129 (Becton Dickinson). The corresponding labeled isotype antibodies served as controls. DCs
130 used for vaccination were washed twice, enumerated, and resuspended in PBS at 1 x 10⁶/mL.

131 We inoculated LM8 cells (5 x 10⁶) in a mouse to make the tumor lysate. After 4 weeks, we
132 resected the tumor mass and soaked the entire tumor in liquid nitrogen to kill the tumor cells.
133 We mixed cryonecrotic tissue with DCs at Culture Day 6, after the tumor was defrosted, and
134 the homogenate was prepared using PBS. The homogenate was passed through a 0.2-µm filter
135 to remove bacteria and tissues and mixed with the DCs for 24 hours.

136 After intraperitoneal injection of 5 mL sodium pentobarbital (Somnopentyl®; Kyontsu
137 Seiyaku, Tokyo, Japan), mice were euthanized by cervical dislocation and their blood was
138 collected. Murine interferon (IFN)-γ and interleukin (IL)-4 release were measured by ELISA
139 using Quantikine® (R & D Systems, Minneapolis, MN) according to the manufacturer's

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

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

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

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

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)

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163 Results

164 We activated antitumor immunity by combining DCs exposed to lysates of cryotreated tumor
165 and reimplantation of the cryotreated primary tumor. On Culture Day 7, the ratio of mature
166 DCs to immature DCs was increased compared with the ratio at Culture Day 6 (Fig. 2;
167 immature DCs, upper left; mature DCs, upper right). Moreover, this increase was more
168 apparent in groups incubated with tumor lysate. Serum IFN- γ levels were greater ($p < 0.0001$)
169 in the mice that received DCs combined with reimplantation of the cryotreated primary tumor
170 (119.0 ± 7.61 pg/mL) than in the cryotreated primary tumor alone group (37.33 ± 2.58 pg/mL).
171 Moreover, the group that received tumor lysate-exposed DCs combined with reimplantation of
172 the cryotreated primary tumor (157.33 ± 14 pg/mL) had a greater ($p < 0.0001$) IFN- γ level
173 than the group that received only tumor lysate-exposed DCs without reimplantation of the
174 cryotreated primary tumor (120.27 ± 11.29 pg/mL) (Fig. 3). Serum IL-4 was lower ($p <$
175 0.0001) in the mice that received DCs exposed to the lysates of cryotreated tumor and
176 reimplantation of the cryotreated primary tumor group (13.33 ± 9.75 pg/mL) than in the
177 excision-only group (45.06 ± 5.71 pg/mL) (Fig. 4).

178 The enhanced immune response by T lymphocytes reduced metastatic lesions. Reduction of
179 the metastatic area was greater ($p < 0.0001$) in the group that received DCs without
180 reimplantation of the cryotreated primary tumor (15.99 ± 3.93 mm²) than in the excision-only
181 group (24.12 ± 3.60 mm²). The reduction of the metastatic area was greater ($p < 0.0001$) in the
182 DCs combined with reimplantation of the cryotreated primary tumor group (5.39 ± 1.49
183 mm²) than in the reimplantation of the cryotreated primary tumor alone group (13.22 ± 2.59
184 mm²) (Fig. 5). CD8(+) T lymphocytes gathered in the pulmonary metastatic area in DC-
185 treated groups, however, NK cells were not recruited to the metastatic area in the DC-treated
186 groups compared with the nonDC-treated groups (Fig. 6). The number of CD8(+) T

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187 lymphocytes per unit area was greater ($p < 0.0001$) in the DCs combined with reimplantation
188 of the cryotreated primary tumor group (8.33 ± 2.57 cells/mm²) than in the reimplantation of
189 the cryotreated primary tumor alone group (2.44 ± 0.53 cells/mm²). Mice that received DCs
190 exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary
191 tumor (12.79 ± 2.14 cells/mm²) showed higher ($p < 0.0001$) levels than the group that
192 received DCs exposed to the lysates of cryotreated tumor without reimplantation of the
193 cryotreated primary tumor (8.71 ± 2.39 cells/mm²) (Fig. 7). The number of NK cells per unit
194 area was greater ($p < 0.0001$) in the group that received DCs exposed to the lysates of
195 cryotreated tumor without reimplantation of the cryotreated primary tumor (3.90 ± 2.17
196 cells/mm²) than in the excision-only group (1.20 ± 0.30 cells/mm²) (Fig. 8). The CD8(+)T
197 lymphocyte, CD4(+) T lymphocyte, and DC infiltrations in reimplanted tumors was similar to
198 that seen with pulmonary metastases (data not shown).

199 **Discussion**

200 Various immunotherapies for osteosarcoma have been tried. As standard treatments for
201 osteosarcoma are ineffectual for many patients, new treatments need to be developed. In the
202 1970s, immunotherapy for osteosarcoma was reported by Southam et al. [42], Neff and
203 Enneking [34], and Campbell et al. [5]. In the 1980s, new methods such as the use of
204 interferons and Bacille de Calmette et Guérin were reported [22, 24, 36]. Another approach
205 used antiidiotypic antibodies using T cells and liposome encapsulation [18, 51, 52]. Current
206 methods of immunotherapy for osteosarcoma include peptide therapy or gene transfer therapy
207 combined with hyperthermia therapy [10, 15, 21, 25, 33]. We asked whether (1) antitumor
208 immunity could be achieved through activation of DCs combined with reimplantation of the
209 cryotreated primary tumor and (2) if metastatic lesions would be prevented owing to enhanced
210 T lymphocytes involvement.

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211 We acknowledge limitations in this study. First, we used mice with an identical genetic
 212 makeup. The structure of the MHC Class I molecules was similar and the T cells could
 213 recognize the MHC Class I. However, we needed to use DCs from a different (albeit
 214 genetically identical) mouse to accomplish our adoptive transfer experiments. We minimized
 215 the potential for an immune response to nonself antigens by using genetically identical tumor
 216 tissue and mice. It would be necessary to use DCs derived from the same individual in
 217 clinical application, but this could not be achieved in our mouse model. In humans, however,
 218 monocytes are separated from the patient's own peripheral blood and DCs can be induced
 219 from these monocytes. Second, we could not completely replicate the clinical approach used
 220 in humans in our mouse model. In clinical cases frozen bone is always returned to the same
 221 site. However, it was impossible to replicate this in our experimental mouse model in which
 222 transplanted tumor cells were removed from the tibia and then returned to the same place after
 223 cryotreatment. In a preliminary experiment we attempted to do just that and these 20 mice
 224 could not move and died of starvation. We therefore used the contralateral gluteal region
 225 flank to check for local recurrence after tumor excision or recurrence from frozen tissue.-
 226 Antitumor immunity appeared activated through DCs combined with reimplantation of the
 227 cryotreated primary tumor or by exposing the transferred DC to lysates of cryotreated tumor.
 228 The use of lymphokine-activated killer (LAK) therapy has been used with other types of
 229 tumors [26]. However, T lymphocytes, which are the effectors, do not accumulate inside
 230 osteosarcoma tumors as expected. Hyperthermia, through autoclaving or pasteurization, with
 231 DCs is thought to enhance the antitumor effect, but hyperthermia causes proteins to denature,
 232 and activation of the antitumor effect is often insufficient [37]. Several studies [12, 31, 41]
 233 report peptide vaccine therapy, but many patients apparently develop immunotolerance [45].
 234 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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235 established definitively although investigations continued to try to overcome the major
236 hurdles associated with immunotherapy (Table 1). We emphasize the immune response is
237 activated by cryotreatment but not by heat-treated tissue. Our method differs from those
238 described by others [7, 9, 10,14]. In some regards DCs are believed the principal APCs for
239 initiating immune responses in vivo [32]. In comparison with other traditional adjunct
240 therapeutic options for cancer, such as radiation therapy and chemotherapy, immunotherapy
241 provides a more targeted treatment to the cancer, with potentially fewer detrimental effects on
242 noncancerous cells [30, 40]. DCs without sufficient cancer antigens may not have the ability
243 to kill tumor cells and present the antigen to T lymphocytes by themselves. Our data suggest
244 the antitumor effect in the group that received DCs without reimplantation of cryotreated
245 primary tumor was almost the same as that in the reimplantation of cryotreated primary tumor
246 alone group. The data further suggest the effects increased only when exposing the DCs to
247 tumor lysates in the absence of cryonecrotic primary tumors. However, combining
248 reimplantation of cryotreated primary tumor and DCs exposed to cryotreated tumor lysates
249 produced synergistic effects. Using reimplantation of cryotreated primary tumor is more
250 appropriate for clinical applications. We therefore believe an efficient immune response will
251 be activated when DCs recognize tumor antigens appropriately. CD8(+) T cells act as an
252 effector by the Th1 route, and this is promoted mainly by IFN- γ and IL-12 [38]. However, IL-
253 4 [21], IL-6, and IL-10 strengthen humoral immunity. Levels of IFN- γ , IL-2, and IL-12
254 generally increase when cell-mediated immunity is activated, and IL-4, IL-6, and IL-10
255 increase when humoral immunity is activated. These cytokines act in opposition to maintain
256 an immune balance.

257 Our data suggest enhanced T lymphocyte recruitment and function reduces metastatic lesions
258 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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259 DCs exposed to lysates of cryotreated tumor combined with reimplantation of the cryotreated
260 primary tumor. NK cells attack the tumor independently of APCs. NK cells attack cells that
261 downregulate MHC Class I expression or have a stressed appearance [44]. We observed a
262 reduced tumor burden in the groups that received transplanted DCs, which correlated with
263 recruitment of CD8 lymphocytes to the tumor site as observed with immunohistochemistry.

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

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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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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.

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

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.

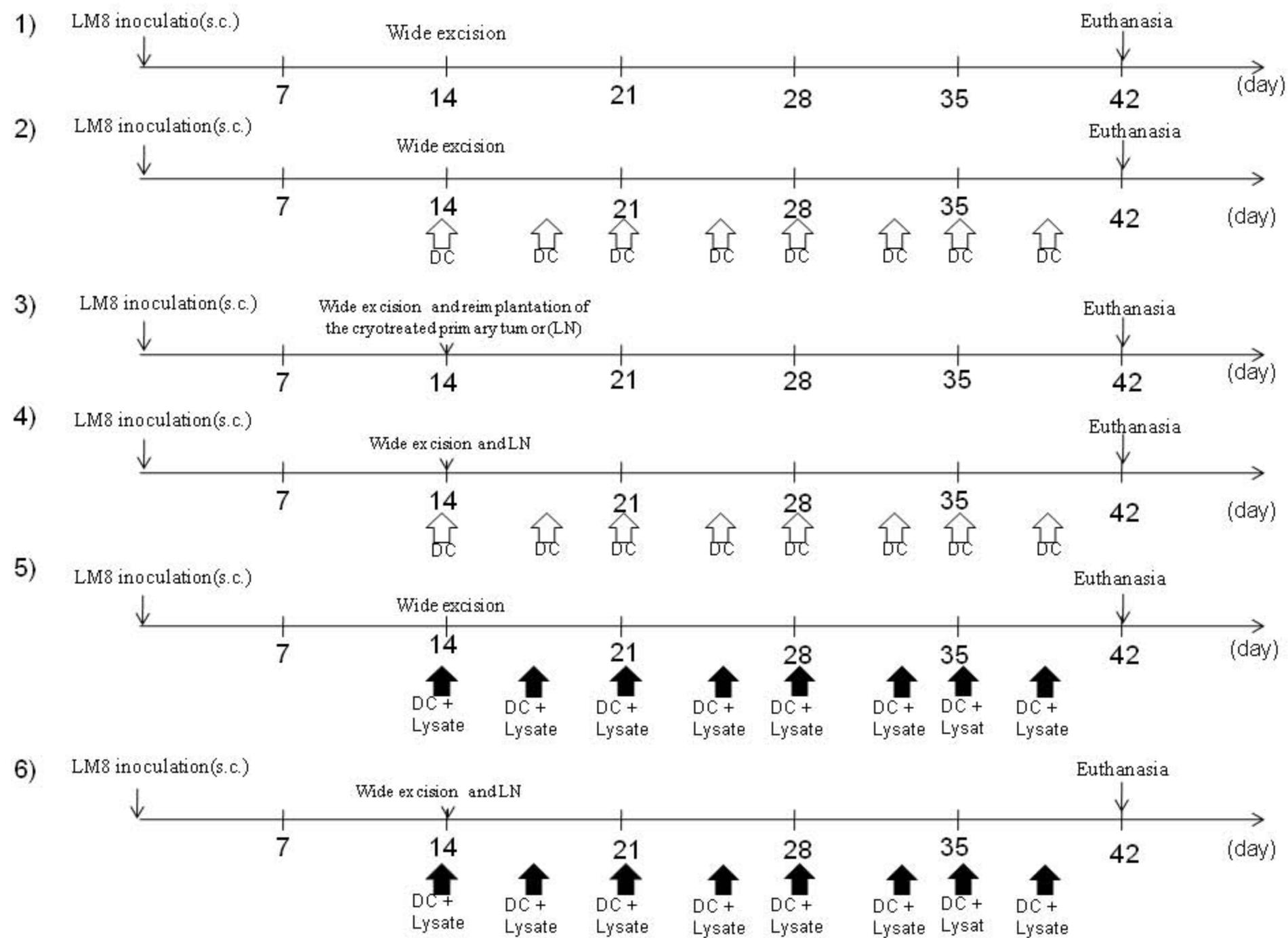
I confirmed this sentence is correct.

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.

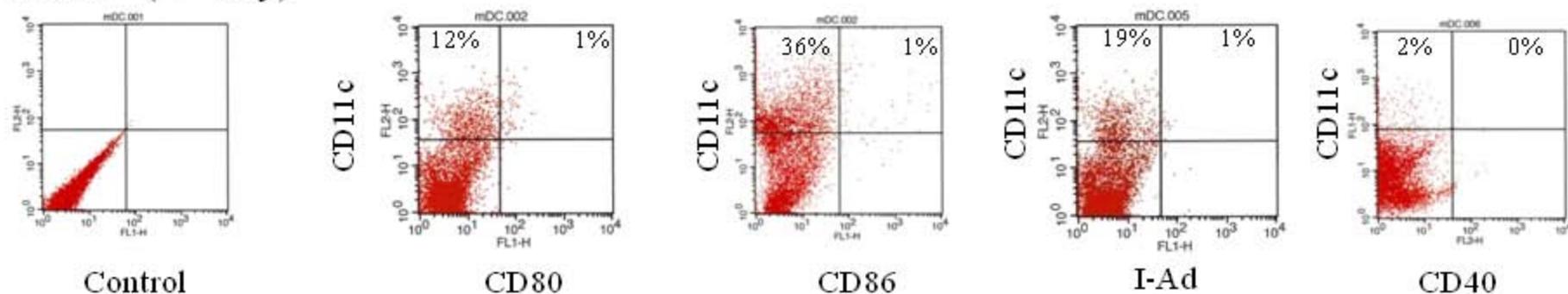
コメント [BP22]: AU: Is this sentence correct as rewritten? Response required.

I confirmed this sentence is correct.

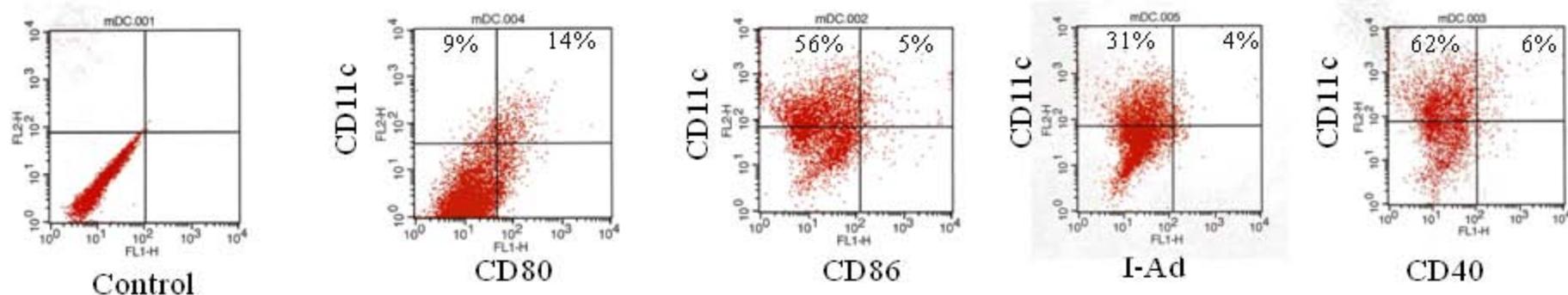
AU: Please do not delete query boxes or remove line numbers; ensure you address each query in the query box.



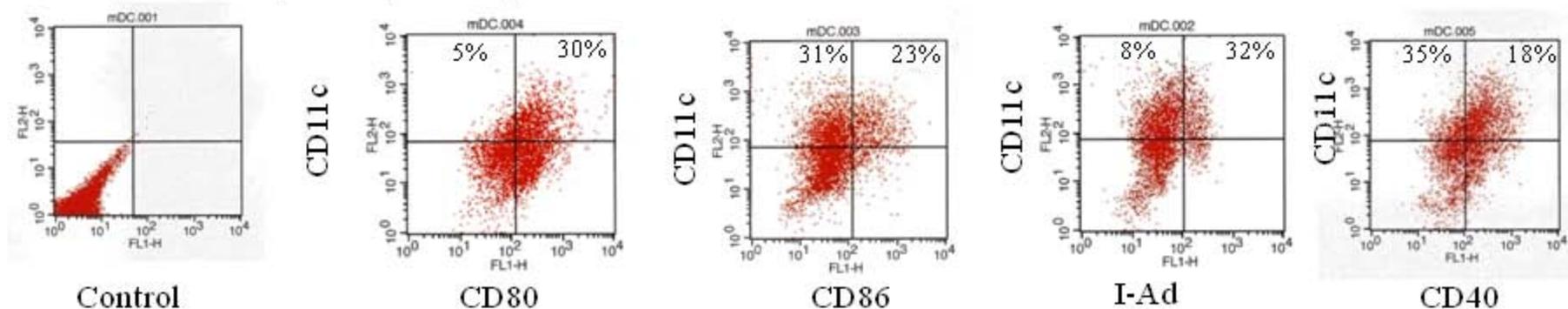
a. DC (6th day)

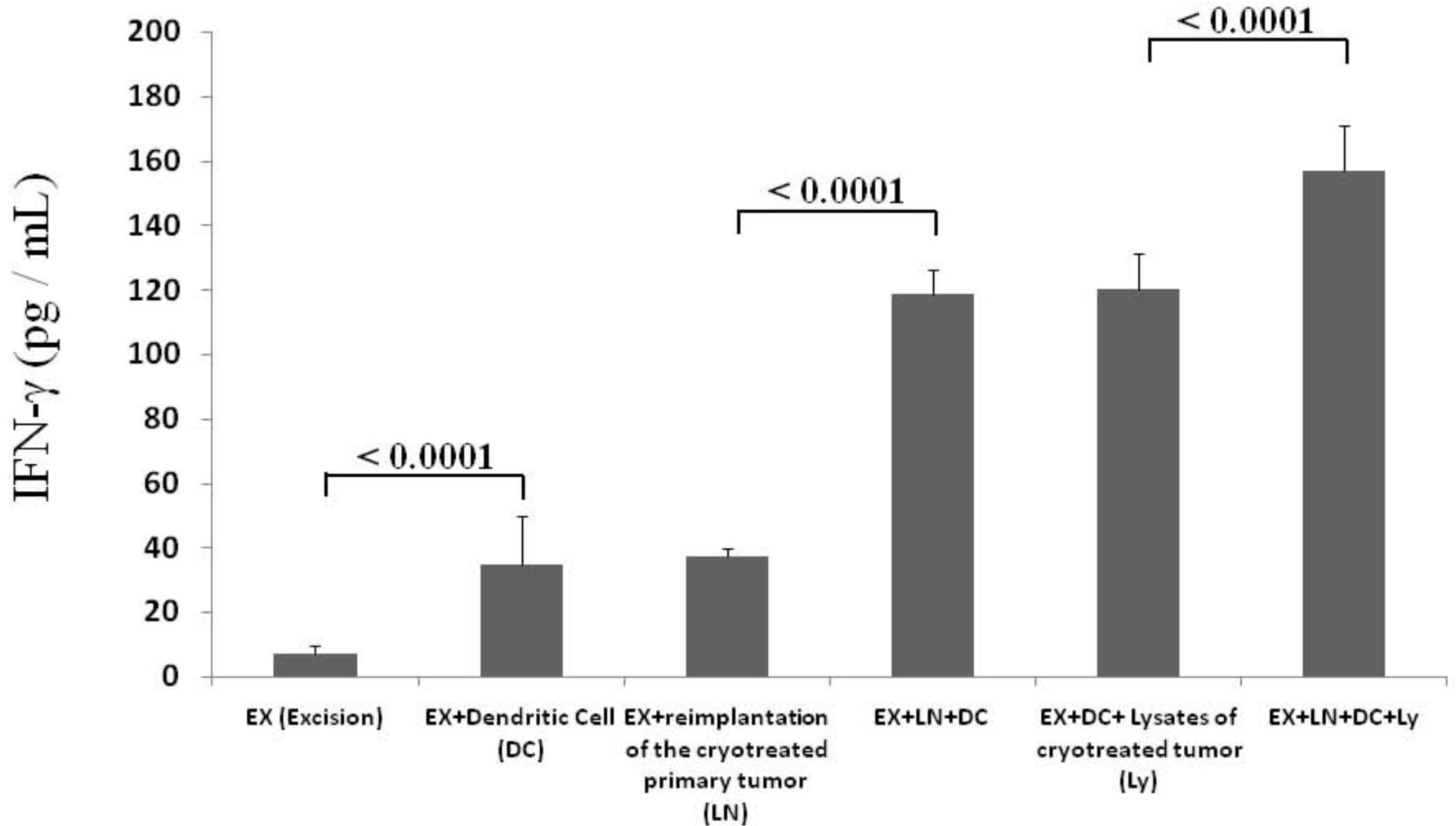


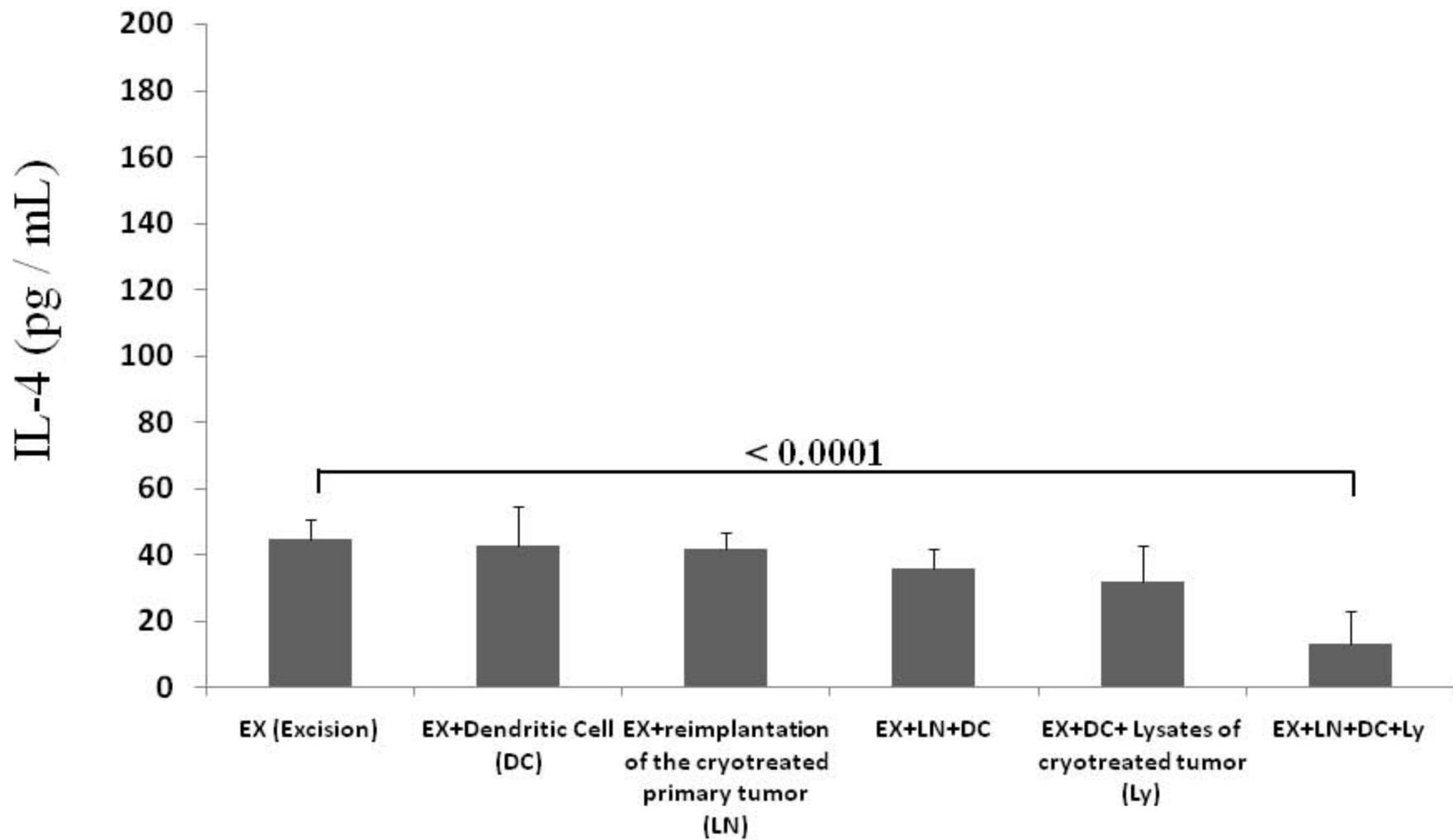
b. DC (7th day)



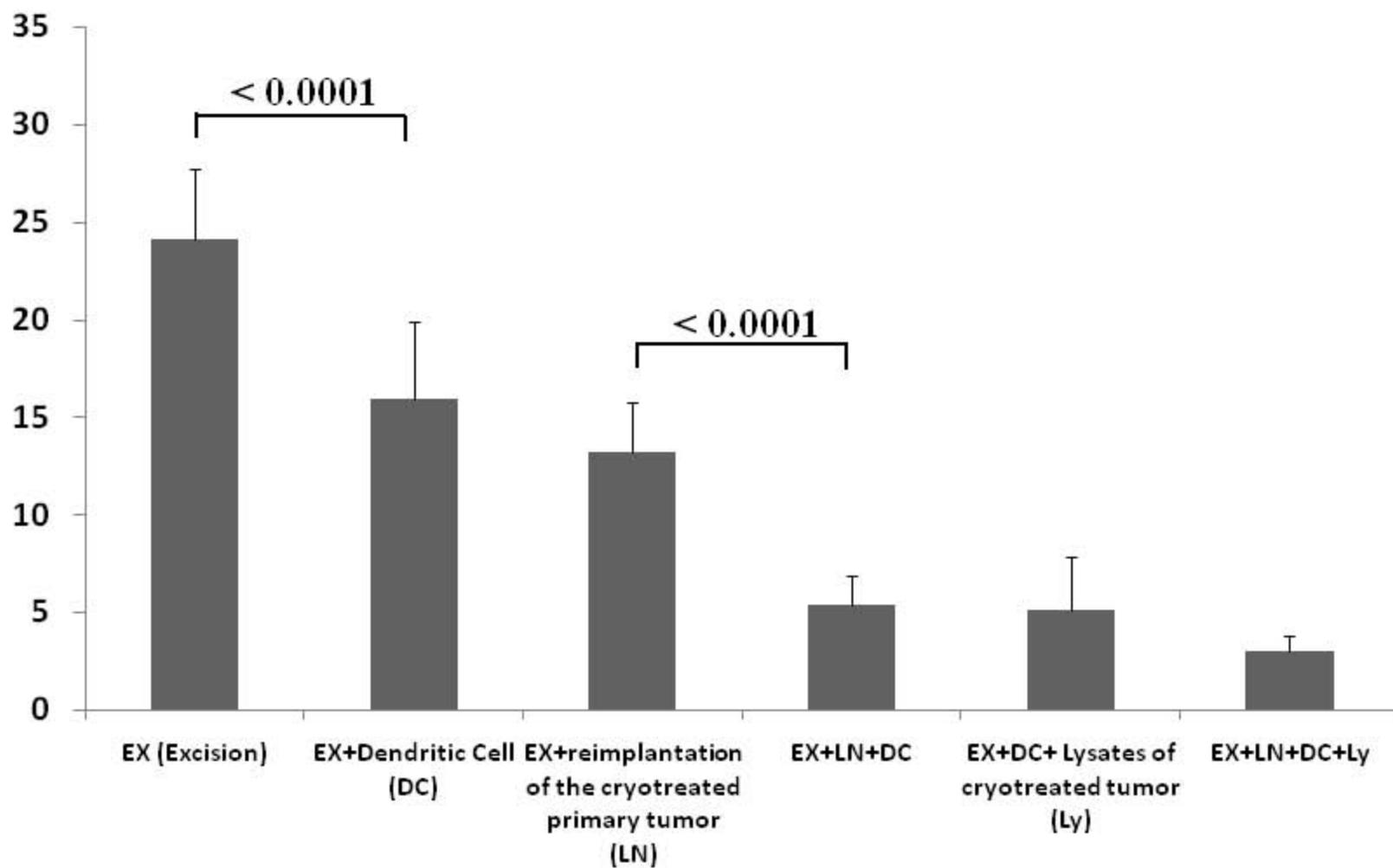
c. DC + Tumor Lysate (7th day)



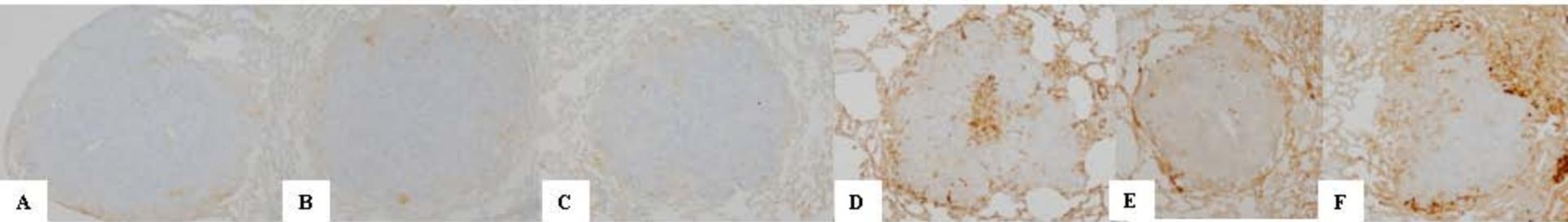




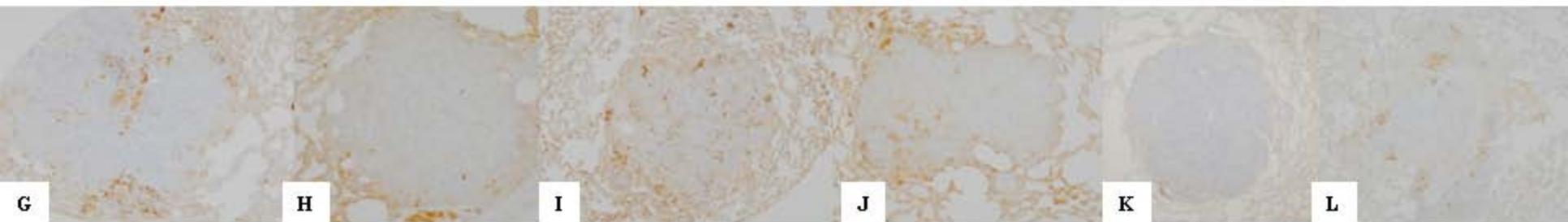
Area of the Lung Metastatic Lesion (mm²)



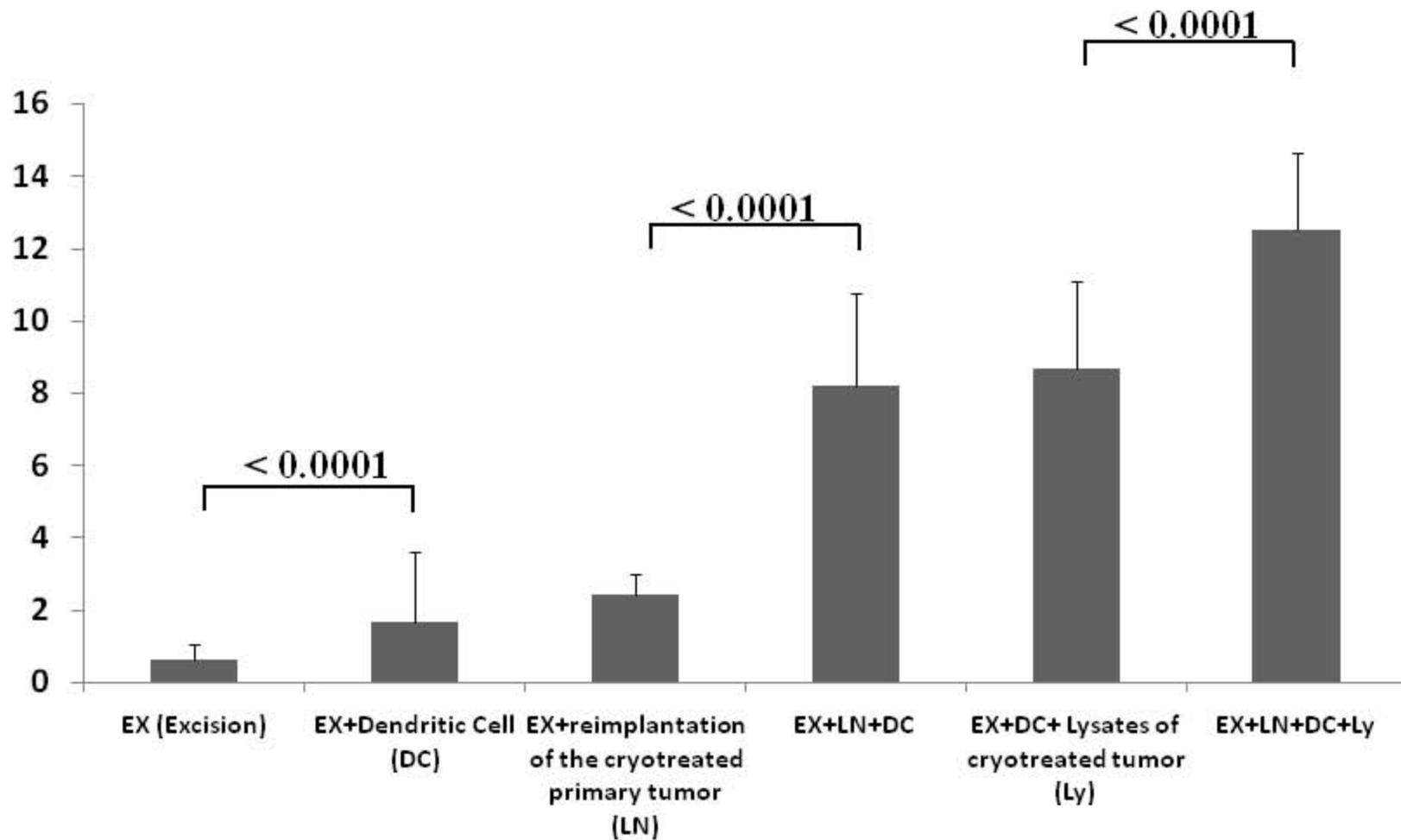
CD8(+) T-Lymphocyte (A~F) (× 200)



NK Cell (G~L) (× 200)



CD8(+) T-Lymphocyte (cells / mm²)



NK Cell (cells / mm²)

