

Enhancement of antitumor immunity by combination of anti-CTLA-4 antibody and radioimmunotherapy through the suppression of Tregs

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Abstract. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed during cluster of differentiation (CD)4⁺ T-cell activation and terminates immune responses by interrupting CD28-enhanced activation. In addition, CTLA-4 is known to be constitutively expressed in regulatory T-cells (Tregs) and to contribute to immune suppression by enhancing the suppressive function of Tregs. However, the molecular mechanisms underlying CTLA-4-mediated Treg suppression remains incompletely understood. Furthermore, it is uncertain whether the *in vivo* immune suppressive functions of CTLA-4 are mediated only by a reduction in the level of conventional T-cell activity, or enhancement of Treg function. The present study demonstrated that combination therapy with an anti-CTLA-4 monoclonal antibody and dendritic cell-mediated radioimmunotherapy (IR/DC) was able to promote an antitumor response and influence Treg function in a mouse model of lung cancer. Cell surface markers, including CTLA-4, CD25 and CD4, were analyzed using flow cytometry, and T-cell activities were measured using ELISPOT and cytotoxicity assays. It was revealed that anti-CTLA-4 combined treatment with IR/DC immunotherapy may execute a more powerful and effective anti-tumor immunity through the inhibition of Treg function.

Introduction

In previous studies, the anti-cytotoxic T lymphocyte antigen (CTLA)-4 antibody was able to enhance the activity of conventional T-cells through the blockade of CTLA-4, which competes with the costimulatory molecule cluster of differentiation (CD) 28 to bind to the ligands CD80 and CD86 on dendritic cells (DCs) (1,2). However, the mechanism by which CTLA-4 influences the suppressive effect of regulatory T-cells (Tregs) *in vivo* remains unclear. Although studies have reported that transgenic CTLA-4 enhanced Treg function and anti-CTLA-4 antibodies induced autoimmune colitis through the suppression of Tregs in a mouse model, the presence of anti-CTLA4 antibodies did not affect the suppressive effect of Treg on CD4⁺CD25⁺ T-cells in mouse gastritis (3,4). Therefore, investigation into the role of CTLA-4 in cancer immunotherapy is required. The present study assessed the inhibition of CTLA-4 using an anti-CTLA-4 monoclonal antibody (mAb), in order to determine whether this inhibition was able to increase the level of antitumor immunity induced by dendritic cell-mediated radioimmunotherapy (IR/DC) in a mouse model *in vivo*. Since malignant cells may exhibit acquired tolerance to tumor-specific antigens and Tregs may be involved in the survival of tumor cells through the suppression of antitumor immunity (5,6), the present study hypothesized that blocking CTLA-4 may break the tolerance and induce antitumor immunity through the inhibition of Tregs.

Materials and methods

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Mice and cancer cell lines. A total of seventy-five male C57BL/6 mice aged 6 weeks and weighing 18–22 g were purchased from Central Lab., Animal Inc. (Seoul, Korea) and the animal experiments were approved by the Dongnam Institute of Radiological and Medical Sciences Institutional Animal Care and Use Committee (Busan, Korea). All mice were maintained in a specific pathogen-free state under a strict light cycle (lights on at 08:00 h and off at 20:00 h) at 21±2°C and 45±10% relative humidity. The Lewis lung carcinoma (LLC) CRL-1642 cells, derived from C57BL/6 mice, were obtained from American

Type Culture Collection (Manassas, VA, USA). The cells were cultured for 7–10 days in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Miami, FL, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Isolation of bone marrow-derived DCs. The C57BL/6 mice (n=5) were sacrificed using CO₂ gas and bone marrow cells were isolated from the tibias and femurs. Red blood cells (RBCs) were lysed by treatment with ammonium-chloride-potassium (ACK) lysing buffer (Gibco; Thermo Fisher Scientific, Inc.) and washed with PBS. The 1x10⁶ cells/ml cells were cultured in 100-mm culture dishes filled with 10 ml RPMI-1640 medium supplemented with 10% FBS, 50 mM 2-mercaptoethanol (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml recombinant mouse granulocyte macrophage colony-stimulating factor (R&D Systems, Inc., Minneapolis, MN, USA) and 10 ng/ml recombinant mouse interleukin-4 (R&D Systems, Inc.) for 3 days. Half of the medium was replaced with fresh medium every day. On the 6th day, the non-adherent cells were collected by pipetting and the surface markers on the cells, such as CD80 (0.2 mg/ml; cat. no., 553769; BD Pharmingen, San Diego, CA, USA), CD86 (0.2 mg/ml; cat. no., 553692; BD Pharmingen), I-A-I-E (0.2 mg/ml; cat. no., 557000; BD Pharmingen), CD11c (0.5 mg/ml; cat. no., 553801; BD Pharmingen) were analyzed using a FC500 flow cytometer (Beckman Coulter, Inc., Miami, FL, USA).

Ionizing radiation (IR) and immunization procedure. The IR and immunization procedures are shown in Fig. 1. Briefly, LLC cells were transplanted subcutaneously into the right thigh (3x10⁵ cells) and left flank (1.5x10⁵ cells) of the mice (12 mice/group). When the diameter of tumor masses reached ~10 mm, IR was applied at a dose of 12 Gy (6 MV photon beam, dose rate of 6.1 Gy/min) to the tumors on the right thighs of the mice using a linear accelerator (Infinity; Elekta Limited, Crawley, UK). Dosimetry was evaluated using an ionization chamber connected to an electrometer system, according to the International Atomic Energy Agency (IAEA) guideline (IAEA TRS-398) (7). Prior to irradiation, the mice were anesthetized with an intraperitoneal injection of 50 mg zoletil (Virbac, Nice, France) plus 5 mg rompun (Bayer Korea, Ansan, Korea) per kg body weight and placed in a customized restraining device and positioned. The irradiation field square was set as 20x20 cm and the radiation was focused on the legs of the mice to minimize whole body exposure. After 24 h, 1x10⁶ cells/100 µl immature DCs were injected into the irradiated tumor for immunization. Immunization was performed 3 times at 1-week intervals. Tumor size was measured twice per week using the following formula: Tumor size (mm²) = length (mm) x width (mm). The 100 µl anti-CTLA-4 mAb (diluted to 2 mg/ml with PBS; catalog no., BE0103; clone 9H10; Bio X Cell, West Lebanon, NH, USA) was administered intraperitoneally to the mice on the same day as every iDC injection.

Flow cytometric analysis. The proportion and number of effector T-cells and CD4⁺CD25⁺ forkhead box P3 (FOXP3)⁺ cells within the total CD4⁺ cell population was evaluated following IR/iDC

combined with anti-CTLA-4 mAb treatment. The C57BL/6 mice (5 mice/group) were sacrificed using CO₂ gas to harvest their tumor tissue and spleens, after 4 days following the final immunization. Tumor infiltrating leukocytes (1x10⁵ cells/ml) were prepared from individual tumor tissues cut with scissors and dissociated using 0.04 mg/ml Liberase (Roche Applied Science, Penzberg, Germany) in RPMI-1640 medium (Welgene, Daegu, Korea) at 37°C for 90 min, followed by passage through a 0.45-µm nylon mesh (BD Pharmingen). Single cell suspensions of splenocytes (1x10⁷ cells/ml) were obtained by grinding the spleens, followed by passage through a 0.45-µm nylon mesh. RBCs were lysed using 1X ACK lysing buffer at room temperature for 2 min. The separated cells were washed with PBS and immunostained with phycoerythrin (PE)-conjugated anti-mouse CD4 (BD Pharmingen, catalog no., 553652) and PE-cyanin7 (Cy7)-conjugated anti-mouse CD25 (BD Pharmingen, catalog no., 552880), respectively, at a concentration of 20 µl/10⁶ cells, in the dark at 4°C for 30 min. The CD4/CD25 double stained cells were resuspended in a 1:4 dilution (total volume 1 ml) of TF Fix/Perm Buffer (transcription factor buffer set; BD Pharmingen; catalog no., 562725) and incubated at 4°C for 40 min in the dark. The permeabilized cells were stained with PE-Cy5-conjugated anti-mouse FOXP3 (eBioscience, CA, USA; catalog no., 15-5773-82) at a concentration of 20 µl/10⁶ cells and were incubated at 4°C for 30 min in the dark. Subsequently, the cells were washed in a 1:5 dilution (total volume 2 ml) of TF Perm/Wash Buffer (transcription factor buffer set; BD Pharmingen; catalog no., 562725). Flow cytometry analysis was performed on a FC500 flow cytometer. Results were generated using CXP v1.0 Software (Beckman Coulter, Inc.).

Enzyme-linked immunospot (ELISpot) assay. ImmunoSpot plates for ELISpot (Merck Millipore) were pretreated with 35% ethanol for 1 min at room temperature. Following the removal of the ethanol, the plates were coated with capture antibodies (10 µg/ml; cat. no., 51-1818KA; BD Pharmingen) overnight at 4°C. The plates were blocked with 10 g/l bovine serum albumin (Sigma-Aldrich; Merck Millipore) for 2 h and washed three times with PBS. Splenocytes (0.5x10⁶ cells/well) and 50 µg/ml LLC cell lysates were added to each well and incubated at 37°C for 24 h. The plate was washed three times with PBS and an additional three times with PBS-Tween buffer, and biotinylated detection antibodies (diluted to 2 µg/ml with PBS; catalog no., 51-1818KA; BD Pharmingen) were added and the plate was incubated for 2 h at room temperature. The plates were washed >3 times with PBS-Tween buffer. Streptavidin-horseradish peroxidase (100 µl per well) was then added, and the plates were incubated for 2 h at room temperature. Subsequent to being washed twice with PBS, a chromogenic substrate and H₂O₂ (AEC substrate; catalog no., 551951; BD Pharmingen) were added to each well to produce visible spots. When adequate spots were developed, distilled water was added to stop the reaction and the plates were air-dried overnight. The number of spots were calculated and the images were analyzed using an EliSpot Reader System (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Cytotoxicity assay. Splenocytes (3x10⁷) isolated from the mice of each group were stimulated by co-culture with

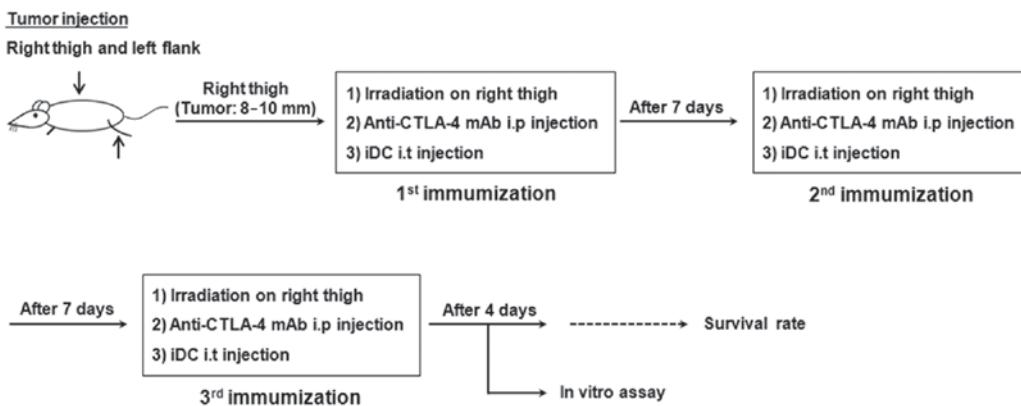


Figure 1. Schematic of the schedule for combination treatment with the anti-CTLA-4 mAb and IR/DC. C57BL6 mice (5 mice/group) were subcutaneously injected with Lewis lung carcinoma cells into the right thigh and the right and left flank. When the tumor grew to ~10 mm, ionizing radiation was applied to the tumor on the right thigh and DCs were injected into the irradiated tumor. IR/DC was performed 3 times at 1-week intervals. The anti-CTLA-4 mAb was administered intraperitoneally to the mice on the same day as every DC injection. IR/DC, dendritic cell-mediated radioimmunotherapy; CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cells; mAb, monoclonal antibody; i.p., intraperitoneal; i.t., intrathecal; iDC, immature dendritic cell.

mitomycin C (Sigma-Aldrich; Merck Millipore) and 10 µg/ml treated LLC cells (3×10^6 cells) for 5 days. The LLC cells were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience, Inc., San Diego, CA, USA) at a concentration of 5 µM for 10 min at 37°C in a humidified incubator with 5% CO₂. Subsequent to labeling, the cells were washed with RPMI-1640 medium containing 10% FBS. Stimulated splenocytes (effector cells) were co-cultured with CFSE-labeled LLC cells (target cells; 2×10^4 /well) at the appropriate effector-to-target cell count ratios (target cell: effect cell, 40:1; 20:1; 10:1) in round-bottomed plates at 37°C in a humidified atmosphere containing 5% CO₂ for 6 h. Subsequent to incubation, the cells were transferred to tubes and placed in an ice water bath. Propidium iodide (50 µg/ml) was added for DNA labeling of dead cells. The dead cells were analyzed using a FC500 flow cytometer. Results were generated using CXP v1.0 Software (Beckman Coulter, Inc., Brea, CA, USA).

Statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison test and log-rank tests. P<0.05 was considered to indicate a statistically significant difference. The software used for statistical analysis was SPSS-18 software (SPSS, Inc., Chicago, IL, USA).

Results

Anti-CTLA-4 mAb enhances the antitumor effect of IR/DC in a mouse model of lung cancer. To evaluate the systemic antitumor effects of anti-CTLA-4 mAb and IR/DC, LLC transplanted mice were treated with single agents or the combination therapy 3 times at 1-week intervals. The growth of distant tumors was significantly suppressed by treatment with IR/DC and combined therapy (Fig. 2A). Single treatment with IR or anti-CTLA-4 mAb did not significantly inhibit distant tumor growth. Similar results were shown in the survival test; combination therapy significantly increased the survival of the mice (Fig. 2B). Although single treatment with anti-CTLA-4 mAb exhibited only a small effect when used alone, this treatment

markedly enhanced the antitumor effect of IR/DC in terms of the tumor growth rate and survival time (Fig. 2). These results suggest that, although the anti-CTLA-4 mAb may be ineffective with respect to inducing antitumor immunity, it may be a good enhancer of immune reponse in other types of immunotherapy.

Ratio of Tregs to CD4⁺ T-cells was decreased in the spleen and tumors following combined therapy. Since it was thought that the function of Tregs was affected by treatment with an anti-CTLA-4 mAb and IR/DC, the ratios of Tregs to CD4⁺ T-cells in the spleens (Fig. 3A) and tumors (Fig. 3B) were measured. In the present study, CD4⁺ T-cells and Tregs were detected using fluorescence-conjugated anti-CD4, anti-CD25 and anti-FOXP3 mAbs (Fig. 3C). The ratios of Tregs to CD4⁺ T-cells were significantly decreased by treatment with IR/DC. Although single treatment with the anti-CTLA-4 mAb did not affect the proportion of Tregs, the Treg proportion was reduced by combination treatment with the anti-CTLA-4 mAb and IR/DC. Decreased ratio of Treg could derived from the decreased number of Treg cells and decreased proliferation of conventional T-cells. Although the decreased ratio of Tregs to CD4⁺ T-cells did not distinguish a reduced number of Tregs from the proliferation of conventional CD4⁺ T-cells, it was certain that decreased ratio of Treg contributed to the induction of antitumor immunity.

Combined therapy decreases the number of Tregs and increases the number of conventional T-cells in the mouse spleen. Following dissection and weighing, the spleens were mashed and the total splenocytes were counted. The number of Tregs per g/spleen was calculated, and the number was significantly decreased following treatment with IR/DC and combined therapy compared to untreated control (Fig. 3D). Single treatment with the anti-CTLA-4 mAb did not significantly alter the number of Tregs. To evaluate the influence of IR/DC and the anti-CTLA-4 mAb on two types of T-cells, total numbers of conventional CD4⁺/CD25⁻ and CD8⁺ T-cells were also calculated. The numbers of CD4⁺/CD25⁻ and CD8⁺ T-cells were significantly increased in the mice following treatment with IR/DC, and the numbers of cells were further increased

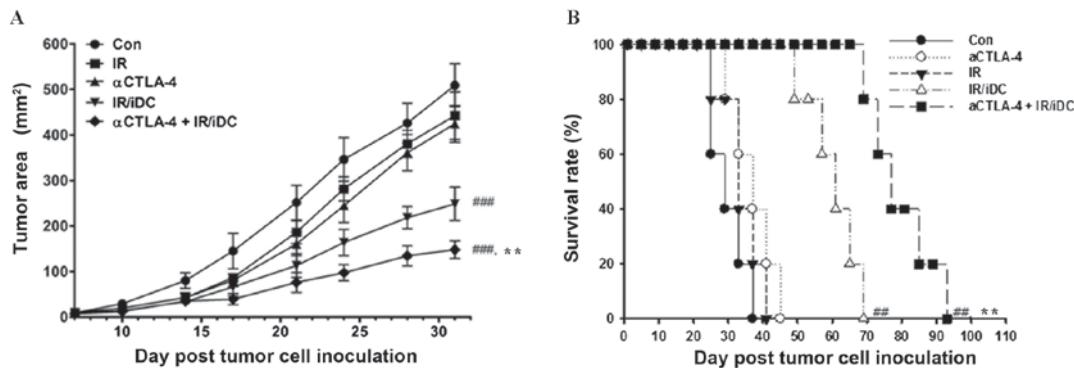


Figure 2. Antitumor effect of anti-CTLA-4 and IR/DC treatment, based on three independent experiments. (A) Tumor size on left flank was measured using a caliper twice per week. (B) Survival rates of the mice are presented as Kaplan-Meier survival curves from an experiment using 5 mice per group. ##P<0.005, ###P<0.001 vs. the Con group. **P<0.005 vs. the IR/DC group. IR/DC, dendritic cell-mediated radioimmunotherapy; DC, dendritic cells; CTLA-4, cytotoxic T lymphocyte antigen-4; Con, control; IR, ionizing radiation.

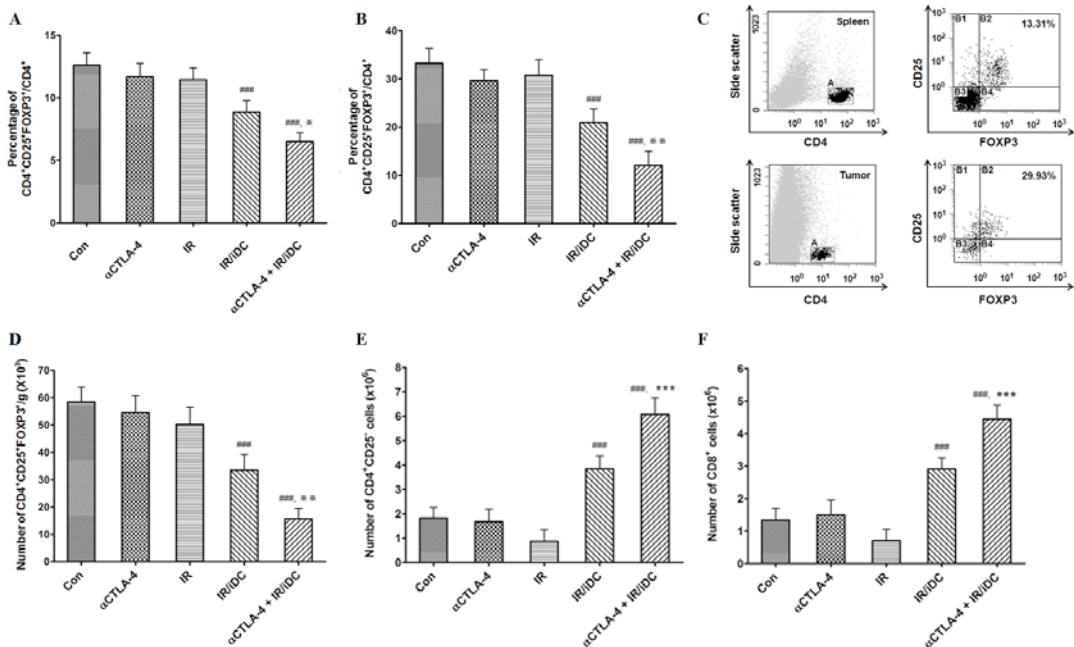


Figure 3. Proportion of Tregs to CD4⁺ T-cells, and the number of Tregs and conventional T-cells in the spleens and tumors of a mouse model of lung disease. Ratios of Tregs to CD4⁺ T-cells in the (A) spleens and (B) tumors were measured. (C) The percentages of CD4⁺ T-cells and Tregs were detected by flow cytometric analysis. (D) The number of Tregs per grams of spleen was calculated following the various treatments. The numbers of (E) CD4⁺/CD25⁺ and (F) CD8⁺ T-cells were assayed following the various treatments. ##P<0.001 vs. the Con group. *P<0.05, **P<0.005 and ***P<0.001 vs. the IR/DC group. IR/DC, dendritic cell-mediated radioimmunotherapy; CTLA-4, cytotoxic T lymphocyte antigen-4; CD, cluster of differentiation; Con, control; IR, ionizing radiation; FOXP3, forkhead box P3; iDC, immature dendritic cell.

by combined therapy with the anti-CTLA-4 mAb and IR/DC (Fig. 3E and F).

Number of tumor-specific T cells is increased following treatment with the anti-CTLA-4 mAb and/or IR/DC, but not subsequent to IR. Since the ratio of reactive T-cells to tumor antigen is small, it is important the absolute quantity of T-cells respond to specific tumor antigens and secrete the tumor protective cytokine, IFN- γ . When tumor antigens reactive T-cells were counted using an EliSpot assay, the number of IFN- γ spots secreted by activated T-cells was significantly increased following treatment with the anti-CTLA-4 mAb alone, IR/DC alone and the combined therapy (Fig. 4). This result suggests that the anti-CTLA-4 mAb and IR/DC

enhance antitumor immunity through an increased number of general immune cells and the clonal expansion of specific T-cells against tumor antigens.

Splenocytes activated by combination therapy are more cytotoxic to tumor cells than splenocytes activated by single agent therapy. In the present study, effective immune responses were revealed to be induced by combination therapy with the anti-CTLA-4 mAb and IR/DC, and immunized splenocytes were shown to effectively destroy tumor cells (Fig. 5). The specific lysis of LLC cells was increased by splenocytes activated by IR/DC compared with inactivated splenocytes, and it was further increased when IR/DC was combined with the anti-CTLA-4 mAb. It was previously

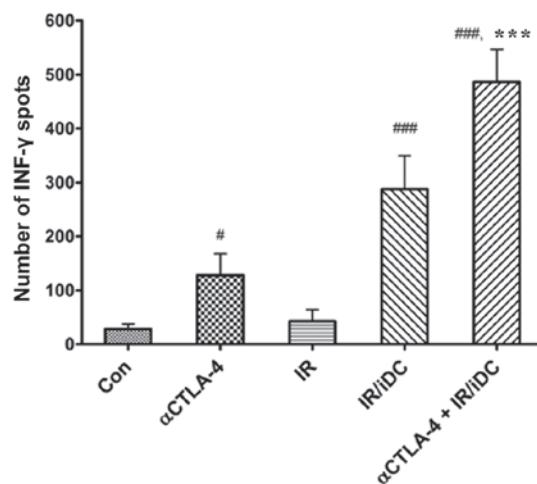


Figure 4. Number of tumor-specific T-cells following treatment with the anti-CTLA-4 antibody and IR/DC. Tumor-specific T-cells were counted using an EliSpot assay and the number of IFN- γ spots secreted by activated T-cells was counted following the various treatments. *P<0.005, **P<0.0005 vs. the Con group. ***P<0.0005 vs. the IR/DC group. IR/DC, dendritic cell-mediated radioimmunotherapy; Con, control; IR, ionizing radiation; CTLA-4, cytotoxic T lymphocyte antigen-4; IFN, interferon.

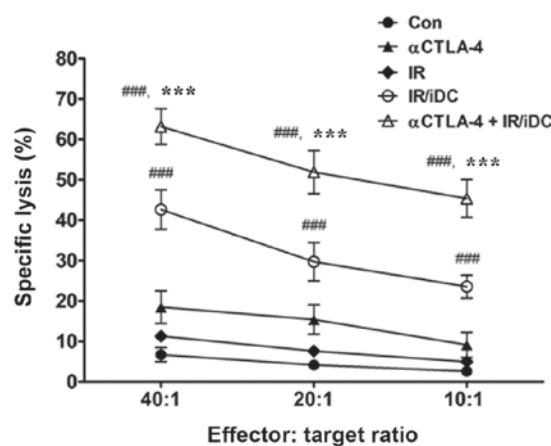


Figure 5. Cytotoxicity of splenocytes on tumor cells following treatment with the anti-CTLA-4 antibody and IR/DC. The tumor cells were co-cultured with splenocytes isolated from mice subsequent to treatment with the anti-CTLA-4 antibody or IR/DC. The specific lysis of the tumor cells was calculated. All experiments were performed in triplicate. ***P<0.0005 vs. the Con group. ***P<0.0005 vs. the IR/DC group. IR/DC, dendritic cell-mediated radioimmunotherapy; CTLA-4, cytotoxic T lymphocyte antigen-4; iDC, immature dendritic cell; Con, control.

suggested that IR/DC is a potent strategy to induce antitumor immunity (8,9). However, the combination of IR/DC with the anti-CTLA-4 mAb may be a more effective for immunotherapy by overcoming an immune checkpoint, CTLA-4.

Discussion

Tregs serve a central role in the maintenance of immune homeostasis and self-tolerance. However, due to their highly suppressive functions, elevated Treg numbers assist the survival of tumors by promoting evasion of immune surveillance (10) and, therefore, represent a major obstacle to successful immunotherapy (11). Following the discovery of the expression

of CTLA-4 on the surface of T-cells, certain functions of CTLA-4 have been hypothesized, including competition with the costimulatory molecule CD28 on CD4 $^{+}$ /CD25 $^{+}$ T-cells for binding to the CD80 and CD86 ligands on antigen presenting cells (APCs), as well as the direct inhibition of APCs and CD4 $^{+}$ /CD25 $^{+}$ T-cells by CTLA-4 and CD80/86 interactions (12). Currently, two types of human anti-CTLA-4 mAbs, ipilimumab and tremelimumab, are being used in the treatment of metastatic melanoma and metastatic mesothelioma (13,14). However, the precise role of CTLA-4 in Tregs during cancer immunotherapy remains unclear. Therefore, an investigation into the role of CTLA-4 on Tregs in cancer immunotherapy was required. The present study revealed that blocking CTLA-4 using an anti-CTLA-4 mAb increased the level of antitumor immunity that was induced by IR/DC, and consequently inhibited tumor growth *in vivo*. However, single treatment with the anti-CTLA-4 mAb was not sufficient to evoke antitumor immunity or a reduction in the number of Tregs despite a slight increase in the number of tumor-specific T-cells.

Since malignant cells may evade early immune surveillance and acquire tolerance to tumor-specific antigens, breaking the acquired tolerance and enhancing antitumor immunity is required for successful immunotherapy (15). Immune check point proteins such as CTLA-4 and programmed cell death protein-1 have been suggested to contribute to the evasion of immune surveillance by tumor cells; thus inhibiting these proteins may enhance antitumor immunity (16,17). The competitive influence of CTLA-4 on conventional T-cells and Tregs have previously been reported (1,3). In addition, an increased number of Tregs has been associated with treatment failure and a poor prognosis of cancer patients. The present study demonstrated that an anti-CTLA-4 mAb inhibited tumor growth *in vivo* through altered Treg function or proliferation.

Although it is known that anti-CTLA-4 mAbs exhibit anti-tumor effects on several types of cancer (18-20), the benefit of this treatment alone was limited until now. However, it was found that anti-CTLA-4 combined treatment with IR/DC immunotherapy may provide a more powerful and effective modality to treat patients with cancer through the efficient reduction of Treg function.

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