Pre-treatment with cyclophosphamide or OX40 (CD134) costimulation targeting regulatory T cell function enhances the anti-tumor immune effect of adoptively transferred CD8+ T cells from wild-type mice

TOMOYUKI UEKI, SATOSHI MURATA, NAOMI KITAMURA, EIJI MEKATA and TOHRU TANI

Department of Surgery, Shiga University of Medical Science, Seta Tsukinowa, Otsu City, Shiga 520-2192, Japan

Abstract. Regulatory T cells (Tregs) are a major obstacle to the establishment of effective cancer immunotherapy. As mediators of immune tolerance, they are a critical target for pre-conditioning for adoptive immunotherapy. Here, we show that pre-treatment with cyclophosphamide or agonistic anti-OX40 mAb augments the anti-tumor immune effect of adoptive CD8+ T cell therapy in a clinically relevant wild-type model, as opposed to a TCR-transgenic mouse model. Tumor antigen-stimulated CD8+ T cells (7×10^6), including a small number (2.17×10^5) of tumor antigen-specific effector CD8+ T cells, were transferred into tumor-bearing mice. A response was detected in the adoptively transferred antigen-specific CD8+ T cells, but was insufficient for the eradication of the established tumor. However, pre-treatment with cyclophosphamide to reduce Tregs was shown to enhance the anti-tumor immune effect of the adoptively transferred CD8+ T cells. Moreover, we demonstrated for the first time that pre-treatment with OX40 costimulation, with the aim of nullifying Treg-mediated suppression, maintained the tumor-specific immune response of adoptively transferred CD8+ T cells, resulting in the eradication of the established tumor. These findings suggest that pre-conditioning with the aim of depleting Tregs is a useful strategy for adoptive cancer immunotherapy.

Introduction

Several strategies for immunotherapy have been evaluated in cancer patients; however, no consistent clinical response has been observed to date. One of the main reasons for this may be an immunological tolerance to tumor (self-) antigens. Tregs play a major role in tolerance to self-antigens. As tumor-associated antigens are derived from self-antigens, Tregs may be responsible for the observed lack of anti-tumor immune

response (1). Indeed, their removal enhances anti-tumor immune response in animal models (1). Thus, one possible strategy for the successful immunotherapeutic treatment of cancer is the depletion of Tregs via pre-conditioning prior to immunotherapy.

Cyclophosphamide is an alkylating chemotherapeutic agent used to treat various types of cancer. Cyclophosphamide decreases the number of Tregs in tumor-bearing rats (2) and inhibits their suppressive abilities (3). Several pre-clinical studies have shown that low doses of cyclophosphamide enhance the anti-tumor activity of adoptively transferred T cells (4-6) or of tumor vaccines (7,8).

OX40 (CD134) is a member of the TNF receptor family that is transiently expressed on effector T cells after T cell receptor (TCR) triggering. OX40 costimulation enhances the effector function, memory development and survival of CD4+ or CD8+ T cells, resulting in the enhancement of anti-tumor immune effects *in vivo* (9-12). OX40 signaling inhibits the Treg-mediated suppression of effector CD4+ T cells without reducing the number of Tregs (13,14), while the OX40-mediated abrogation of Treg function boosts adoptive immune response and increases tumor rejection (15). However, little is known regarding the efficacy of pre-treatment using OX40 costimulation followed by adoptive T cell therapy on Treg-mediated suppressive function.

Clonal CD8+ T cells isolated from tumor antigen-specific TCR-transgenic mice have generally been used in murine models of adoptive T cell therapy. This is because a number of tumor antigen-specific T cells can be obtained from these mice, and the response of the adoptively transferred T cells can easily be detected in the recipients, whereas from wild-type mice it is difficult to obtain a sufficient number of Ag-specific CD8+ cells for the detection of immune effect. However, for the clinical application of new strategies for adoptive T cell therapy, an evaluation of anti-tumor immune effect using adoptively transferred T cells from wild-type animals is, if feasible, preferable. The present study evaluated the availability of antigen-specific CD8+ T cells from wild-type tumor-bearing mice for adoptive T cell therapy of an established tumor. The results revealed that pre-treatment with cyclophosphamide or OX40 costimulation to inhibit Treg-mediated suppressive function promoted the effector function of CD8+ T cells adoptively transferred from tumor-bearing wild-type mice, and

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Correspondence to: Dr Satoshi Murata, Department of Surgery, Shiga University of Medical Science, Seta Tsukinowa, Otsu City, Shiga 520-2192, Japan
E-mail: satoshi1@belle.shiga-med.ac.jp

Key words: murine model, T cells, costimulation, tumor immunity

resulted in the eradication of the established tumor in the recipient mice.

Materials and methods

Animals and cell lines. FVB/N mice were commercially obtained from The Jackson River Laboratory (USA). The animals were housed under pathogen-free conditions at the Shiga University of Medical Science, Japan. Experiments involving the use of the mice were performed in accordance with protocols approved by the Animal Care and Use Committee of Shiga University of Medical Science.

Non-transgenic (NT) cells were derived from the spontaneous mammary tumors of female HER2/neu transgenic mice as previously described (16). This NT cell line stably overexpresses rat HER2/neu cDNA. NT cells were grown in defined breast media and maintained at 37˚C in 5% CO_2. NIH-3T3 cells were grown in 3T3 media at 37˚C in 10% CO_2. 3T3 neu cells derived from NIH-3T3 cells overexpressing rat HER2/neu proto-oncogene were grown in 3T3 media with 0.3 μM methotrexate at 37˚C in 10% CO_2. The 3T3 neu cells were genetically modified to express murine cytokine GM-CSF using retroviral vector MFG as previously described (9,16,17), resulting in a 3T3-neu/GM cell line.

Antibodies and reagents. The RNEU$_{420-429}$ (PDSLRDLSVF) and NP$_{118-126}$ (RPQASGVYM) peptides (>95% purity) were synthesized and generously donated by the Oncology Peptide Synthesis Facility at Johns Hopkins University. Agonistic anti-OX40 mAb was produced from OX86 hybridoma cell lines. Purified rat IgG was used as a control Ab (Sigma). The antibodies were reconstituted in PBS, and were administered by i.p. injection at a dose of 300 μg per mouse in 400 μl PBS. APC anti-mouse CD8a, PE rat anti-mouse IFN-γ, FITC anti-mouse CD4 and PE anti-mouse CD25 were obtained from BD Pharmingen. APC-conjugated anti-mouse/rat Foxp3 and Foxp3 staining buffer sets were obtained from eBioscience. Image cytometry on sections (ICS) was performed as previously described (7) using a Cytofix/Cytoperm™ Plus (with Golgistop™) kit from BD Biosciences. Cyclophosphamide obtained from Sigma Chemical Co. was reconstituted in 400 μl PBS, and administered by i.p. injection at a dose of 100 mg/kg body weight. CD8$^+$ T cells were isolated from splenocytes and lymphocytes by magnetic separation using Dynabeads FlowComp™ mouse CD8 (Invitrogen). Cells were collected using the BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using Cell Quest (BD Biosciences) and FlowJo (Tree Star Inc.) software.

Immunization of donor mice. Female FVB mice (6-8 weeks old) were injected s.c. with 5x10^6 NT2.5 tumor cells in the mammary fat pad on day 3, and with 3x10^6 3T3 neu/GM vaccine cells divided equally between two forelimbs and one hind limb on day 0. T cells were isolated from splenocytes and lymphocytes by nylon wool columns on day 7. These cells were incubated at 37˚C in 5% CO_2 for 2 days with T2D^q cells pulsed with RNEU$_{420-429}$ at a responder to stimulator ratio of 5:1. After 2 days of stimulation, the CD8$^+$ T cells were isolated by magnetic separation using Dynabeads FlowComp mouse CD8. The purity of CD8$^+$ T cells was confirmed to be >95%.

Adoptive immunotherapy. The FVB recipient mice were injected s.c. with 5x10^6 NT2.5 tumor cells in the mammary fat pad on day 3, and i.p. with 100 mg/kg cyclophosphamide, 300 μg anti-OX40 mAb or 300 μg control IgG on day 2. Seven million HER2/neu-primed CD8$^+$ T cells suspended in 400 μl PBS were adoptively transferred into the recipient mice on day 0. Splenocytes and lymphocytes were harvested from the recipient mice on day 6, and ICS was performed. Tumor size was measured and recorded every 3 or 4 days according to the diameter along the orthogonal axes.

Population of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in tumor-inoculated mice following cyclophosphamide or anti-OX40 mAb administration. FVB mice were injected s.c. with 5x10^6 NT2.5 tumor cells in the mammary fat pad on day 1, and i.p. with 100 mg/kg cyclophosphamide, 300 μg anti-OX40 mAb or 300 μg control IgG on day 0. Splenocytes and lymphocytes were harvested, and red blood cells were lysed on days 0, 2, 4 and 6. Subsequently, the population of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells was investigated using a flow cytometer.

Statistical analysis. Data analysis was performed using the Student's t-test.

Results

Generation and expansion of HER2/neu-specific CD8$^+$ T cells for adoptive immunotherapy. As described above, for clinically relevant immunotherapy it is more relevant to use a wild-type mouse model than an antigen-specific TCR-transgenic mouse model when evaluating anti-tumor efficacy. However, these mice have a lower number of Ag-specific CD8$^+$ T cells, and in adoptive cytotoxic T lymphocyte (CTL) therapy for tumor eradication, the efficacy of the anti-tumor immune effect is dependent on the number of tumor-antigen-specific CTLs transferred; the greater the number of CTLs, the greater the efficacy. Consequently, antigen-specific CD8$^+$ T cells were generated in vaccinated tumor-bearing wild-type mice and expanded with antigen-specific re-stimulation *in vitro*.

HER2/neu-specific CD8$^+$ T cells, which were confirmed by IFN-γ-producing CD8$^+$ T cell response to RNEU$^{420-429}$, a HER2/neu immnodominant peptide (16), were generated in the tumor-bearing FVB/N wild-type mice treated with HER2/neu-targeted vaccine (16,17). T cells from these vaccinated FVB/N wild-type mice were re-stimulated with T2D^q cells pulsed with RNEU$_{420-429}$. Subsequently, the HER2/neu-specific CD8$^+$ T cells were expanded. The maximum frequency of HER2/neu-specific CD8$^+$ T cells was 3.14±0.276% on day 2 after re-stimulation *in vitro* (Fig. 1). Consequently, CD8$^+$ T cells re-stimulated with RNEU$_{420-429}$ for 2 days *in vitro* were used for the adoptive transfer experiments.

Persistence of effector function of adoptively transferred CD8$^+$ T cells with pre-conditioning for Treg depletion. A total of 7x10^6 *in vitro* re-stimulated CD8$^+$ T cells from vaccinated wild-type FVB/N mice were adoptively transferred into tumor-bearing recipient FVB/N mice with control rat-IgG. More HER2/neu-specific CD8$^+$ T cells were detected in tumor-bearing recipient mice with T cell transfer than in tumor-bearing mice without T cell transfer (Fig. 2A), indicating that
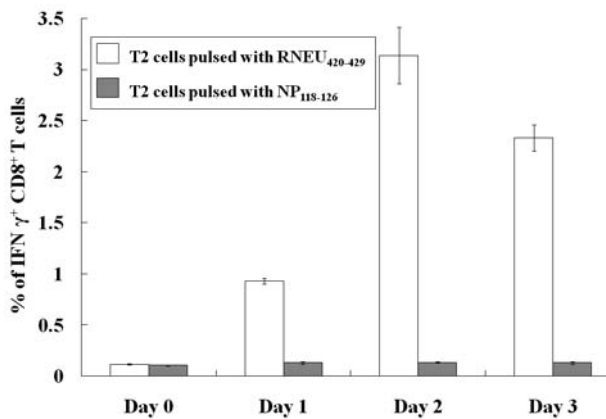
Figure 1. Isolated T cells from immunized mice were activated by the stimulation of T2D^q cells pulsed with RNEU$_{420-429}$. Representative data of three individual experiments are shown. There were three mice in each group. Error bars represent the SD of triplicate assays.

a small number (~2.17x10^5 cells) of HER2/neu-specific CD8$^+$ T cells persisted in an immune response to HER2/neu in the tumor-bearing recipients.

The recipient mice were pre-conditioned with cyclophosphamide or anti-OX40 mAb to inhibit the Treg-mediated suppression of T cells. Antigen-primed CD8$^+$ T cells were transferred 2 days after pre-conditioning, and the persistence of antigen-specific CD8$^+$ T cells was observed 6 days after the adoptive transfer of CD8$^+$ T cells. T cell-transferred recipient mice pre-treated with cyclophosphamide administered i.p. retained significantly more HER2/neu-specific CD8$^+$ T cells than the mice without pre-treatment (Fig. 2B). T cell-transferred recipients pre-treated with anti-OX40 mAb administered i.p. also retained significantly more HER2/neu-specific CD8$^+$ T cells than the mice without pre-treatment (Fig. 2C). Tumor-bearing mice treated with cyclophosphamide or anti-OX40 mAb did not generate HER2/neu-specific CD8$^+$ T cells (Fig. 2B and C). This indicates that the frequency and function of a small number of adoptively transferred HER2/neu-specific CD8$^+$ T cell clones were maintained *in vivo*.

Enhanced anti-tumor immune effects of adoptively transferred HER2/neu-specific CD8$^+$ T cells with the pre-conditioning of Tregs in established tumor-bearing recipient mice. The anti-tumor efficacy of adoptive CTL therapy was examined in the tumor-bearing recipients with or without pre-treatment for the inhibition of Treg-mediated suppression. The recipient mice with adoptively transferred CD8$^+$ T cells alone exhibited significantly delayed tumor growth in comparison to the mice without treatment (IgG i.p. alone). However, alone the adoptively transferred CD8$^+$ T cells were incapable of eliminating the tumors entirely (Fig. 3). Mice pre-treated with cyclophosphamide alone exhibited significantly inhibited tumor growth in comparison with the mice without pre-treatment. In contrast, mice pre-treated with anti-OX40 mAb alone did not exhibit anti-tumor effects. This suggests that cyclophosphamide acts as an anti-tumor agent, while anti-OX40 mAb has no anti-tumor efficacy on its own (Fig. 3). However, mice pre-treated with cyclophosphamide or anti-OX40 mAb into which CD8$^+$ T cells were then adoptively transferred demonstrated significantly inhibited tumor growth
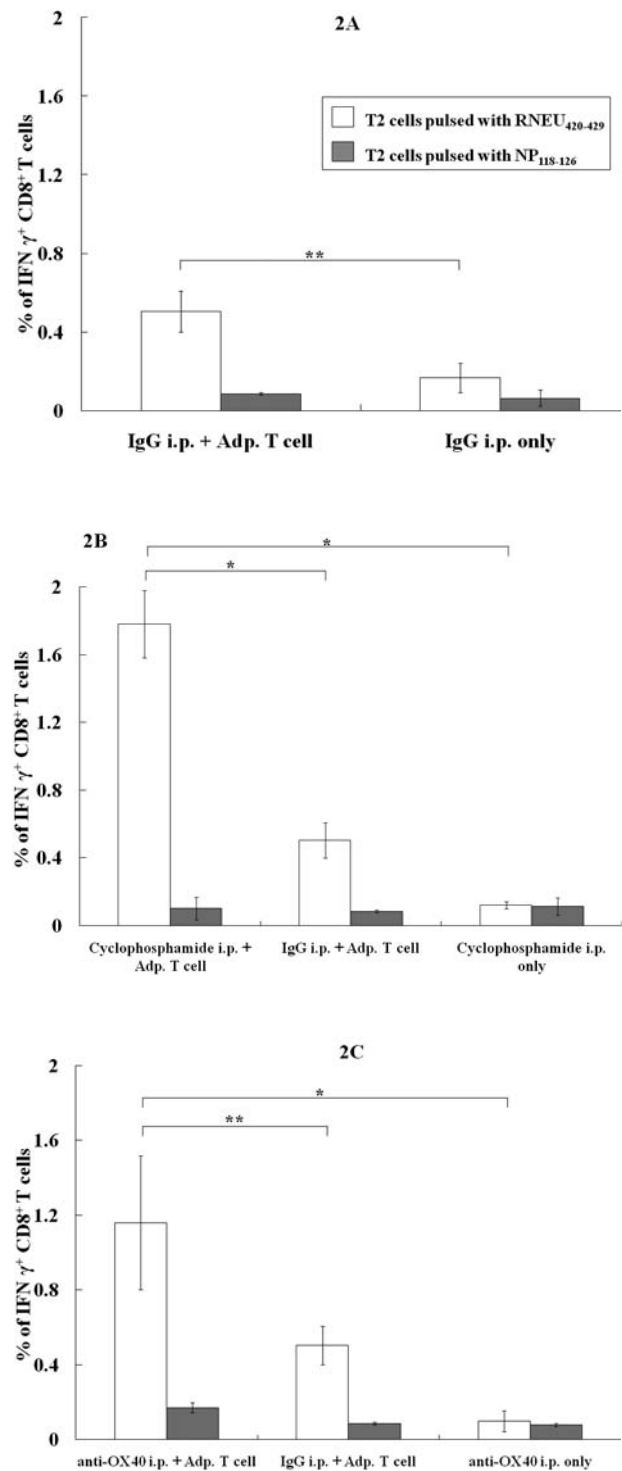

Figure 2. Pre-treatment with cyclophosphamide or anti-OX40 mAb augments the immune effects of adoptively transferred T cells. Representative data of three individual experiments are shown. There were three mice in each group. Significance was evaluated using the Student's t-test. *P<0.01; **P<0.05.

in comparison to the mice that received the adoptive transfer of CD8$^+$ T cells alone. In the cyclophosphamide or anti-OX40 mAb pre-treated mice, the established tumors were eventually completely eliminated (Fig. 3). Thus, pre-conditioning with cyclophosphamide or anti-OX40 mAb appears to synergistically enhance the function of a small number of tumor antigen specific CTLs from wild-type tumor-bearing hosts (~2.17x10^5 cells) to eradicate the established tumor.
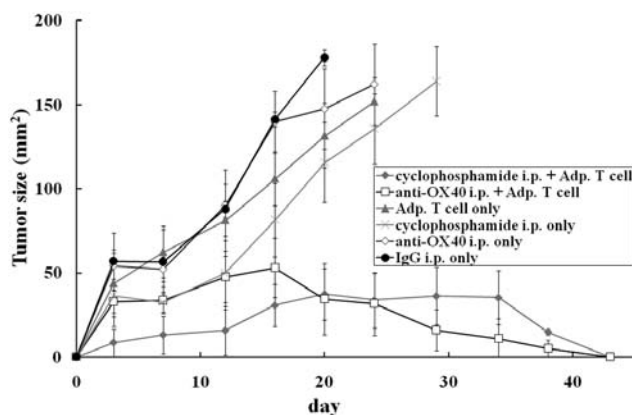
Figure 3. Adoptively transferred HER-2/neu-primed CD8+ T cells with cyclophosphamide or anti-OX40 mAb pre-treatment retard tumor growth. Representative data of three individual experiments are shown. The mean tumor size per group (n=3) ± SD is shown.
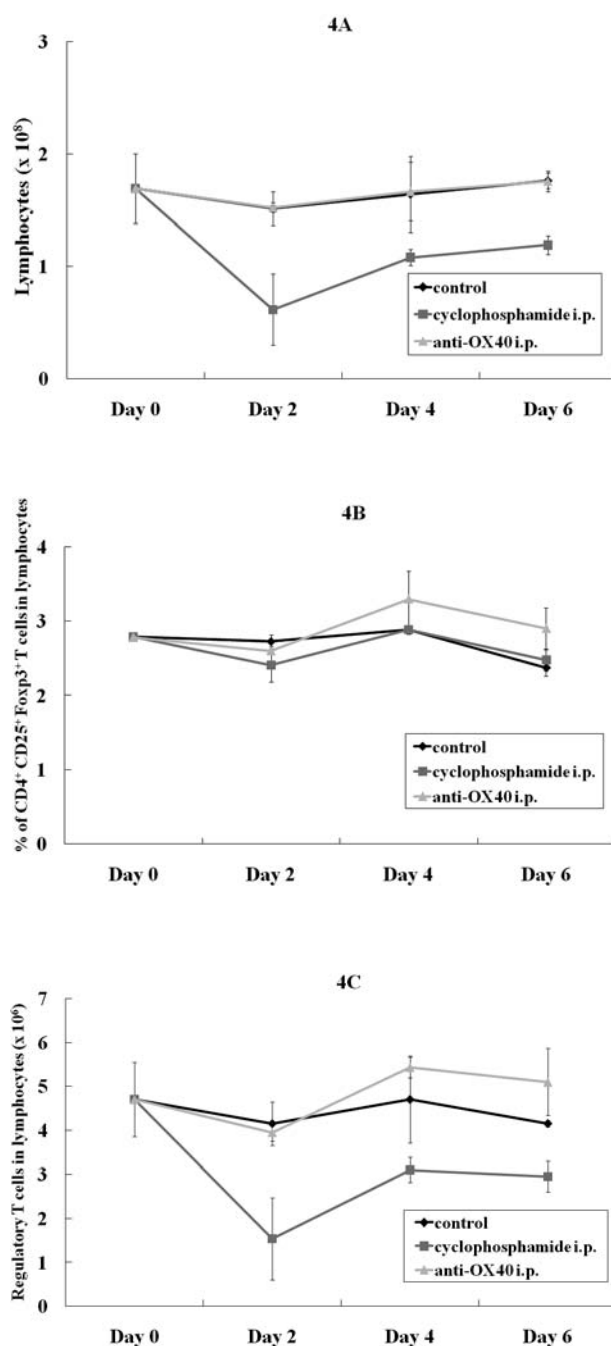

Figure 4. Effects of cyclophosphamide or anti-OX40 mAb administration on the number of Tregs. Representative data of three individual experiments are shown. There were three mice in each group. Error bars represent the SD of triplicate assays.

Frequency of Foxp3+ Tregs after cyclophosphamide or anti-OX40 mAb administration. The number of Tregs after the administration of cyclophosphamide or anti-OX40 mAb was evaluated. The total number of lymphocytes was found to be decreased after cyclophosphamide administration, but remained constant after anti-OX40 mAb administration (Fig. 4A). The percentage of CD4+CD25+Foxp3+ Tregs in lymphocytes was constant after the administration of cyclophosphamide or anti-OX40 mAb (Fig. 4B). The number of CD4+CD25+Foxp3+ Tregs in lymphocytes from the spleen or lymph nodes showed a maximum decrease on day 2 after cyclophosphamide administration (Fig. 4C). In contrast, the number of CD4+CD25+Foxp3+ Tregs in lymphocytes was fixed after anti-OX40 mAb administration (Fig. 4C). However, though anti-OX40 mAb did not reduce the number of Tregs, it nonetheless appeared to inhibit Treg-mediated suppressive function.

Discussion

The results of the present study highlight two important findings related to adoptive immunotherapy. First, pre-treatment with cyclophosphamide or anti-OX40 mAb targeting Tregs can maintain adoptively transferred tumor antigen-specific CD8+ cells in tumor-bearing mice, resulting in enhanced anti-tumor effects. Second, the eradication of established tumors can be obtained *in vivo* with the inhibition of Tregs by small numbers of adoptively transferred tumor antigen-specific CD8+ T cell clones from vaccinated tumor-bearing wild-type mice, as opposed to the tumor antigen-specific TCR-transgenic mouse model commonly used. This suggests that T cell therapy with pre-conditioning is a potential clinically relevant form of immunotherapy.

Several strategies for immunotherapy in cancer patients have been evaluated; however, no significant clinical response has been observed to date. In mice, the depletion of immune cells by radiation or chemotherapy before adoptive T cell therapy can improve the anti-tumor efficacy of transferred CD8+ T cells (18). Several mechanisms might underlie the augmented efficacy of tumor-reactive T cells in the lympho-penic environment, including the elimination of immuno-suppressive cells such as Tregs, the depletion of endogenous cells that compete for activating cytokines, and the increased function of antigen-presenting cells.

Tregs are crucial for the maintenance of peripheral self-tolerance and for the suppression of anti-tumor response (19). The presence of Tregs may hinder the development of anti-tumor immune response following adoptive immunotherapy. Therefore, one possible strategy for augmenting adoptive T cell therapy may be to focus on the abrogation of the activity of the host Tregs prior to immunotherapy treatment. Several studies have shown that a low dose of cyclophosphamide can enhance the anti-tumor activity of adoptively transferred

T cells. It has recently been demonstrated that cyclophosphamide decreases the number of Tregs while inhibiting their suppressive capability (20). In the current study, cyclophosphamide decreased both the number of lymphocytes and Foxp3^{+} Tregs. Under such lymphopenic conditions, residual naïve T cells may proliferate and thus reconstitute a nearly normal lymphocyte pool, a process known as homeostatic proliferation (3). Tumor cells produce suppressive cytokines such as TGF-ß, which directly expand pre-existing Tregs and convert naïve T cells into Tregs. Thus, depleted Tregs can be replenished (15,21-24). Regarding the TCR repertoire, it is conceivable that any available antigens from normal tissues or pathogens beside tumor antigens could lead cognate lymphocytes to Treg cells through conversion, rather than to effector T cells. This allows the peripheral generation of Treg cells with a wide TCR repertoire, increasing their chances of encountering the antigen that activates their suppressive function (24). In the current study, a single administration of tumor antigen-specific T cells with the pre-treatment of cyclophosphamide was capable of eliminating a tiny established tumor that had been challenged 1 day before cyclophosphamide administration and 3 days before T cell transfer. In cases where repeated pre-conditioning by cyclophosphamide is required for an established larger tumor, the replenishment of Tregs with a wide TCR repertoire after their depletion might pose a problem.

Considering the replenishment of Tregs with a wide TCR following the depletion of Tregs, the functional inactivation of Tregs without T cell reduction would be an ideal strategy for Treg-targeted immunotherapy. OX40 triggering inhibits Treg-mediated suppressive functions (13-15), and OX40 costimulation reduces Foxp3 protein expression on Tregs. This reduced Foxp3 expression correlates with the abrogation of the Treg-mediated suppression of anti-tumor immunity (data not shown). In the present study, the number of lymphocytes and Tregs remained stable after OX40 costimulation (Fig. 4), and adoptively transferred HER2/neu-primed CD8^{+} T cells maintained their anti-tumor response in the mice pre-treated with anti-OX40 mAb. The finding that OX40 signaling on Tregs leads to their functional inactivation without a reduction in their numbers suggests that OX40 could augment the anti-tumor immune effects of adoptive T cell therapy without inciting the replenishment of Tregs. OX40 costimulation enhances OX40-expressing CD8^{+} T cell function directly, as well as by means of CD4^{+} T cells (9). In the present experimental setting, in addition to the direct inhibition of Treg-mediated suppression by OX40 costimulation, it is possible that OX40 signaling may have concomitantly increased a direct enhancement of adoptive CD8^{+} T cell function. Anti-OX40 mAb administered 2 days before T cell transfer might persist in its activity and may costimulate OX40-expressing effector CD8^{+} T cells activated by HER2/neu-peptide *in vitro* prior to the transfer. These unique functions of OX40 costimulation suggest a new strategy for adoptive T cell therapy targeting both Tregs and effector T cells by a sequence administration of anti-OX40 mAb before and after effector T cell transfer.

In general, large numbers of clonal CD8^{+} T cells isolated from antigen-specific TCR-transgenic mice are used for evaluating the effects of several types of murine adoptive immunotherapy. However, in human immunotherapy, it is difficult to obtain a large number of tumor antigen-specific CTLs, even if they are expanded by peptide-pulsed dendritic cells or anti-CD3 Ab. It is therefore important to develop an effective strategy to maintain and improve the function of the limited number of tumor antigen-specific CD8^{+} T cells that are adoptively transferred for anti-tumor immunotherapy. In the present study, adoptively transferred tumor antigen-specific CTLs alone had a very limited anti-tumor immune effect and did not succeed in eliminating the established tumors. However, pre-treatment with cyclophosphamide or anti-OX40 mAb for the inhibition of Treg-mediated suppression maintained and improved the function of a small number of adoptively transferred antigen-specific CD8^{+} T cells from vaccinated tumor-bearing wild-type mice. These findings indicate that pre-conditioning with cyclophosphamide or anti-OX40 mAb for the inhibition of Treg-mediated suppression followed by adoptive T cell therapy synergistically enhances anti-tumor immune effects in a tumor-bearing host.

In conclusion, in this clinically relevant model using a small number of adoptively transferred CTLs from wild-type mice, a tumor antigen-specific response was detectable in recipient tumor-bearing mice. Pre-conditioning with cyclophosphamide or OX40 costimulation to inhibit regulatory T cell-mediated suppression synergistically enhanced the anti-tumor immune response of the CTLs. These combined treatments eradicated the established tumors, suggesting that pre-treatment targeting Tregs is useful for adoptive T cell immunotherapy.

Acknowledgements

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