

## Eradication of Established Murine Skin Tumors by Cyclic Chemoimmunotherapy with Cyclophosphamide and Effector T Cells

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### Abstract

**Background:** Nonmyeloablative chemotherapy followed by adoptive immunotherapy is an attractive strategy for depleting regulatory T cells in the host. However, its efficacy is transient. Here, we aim to investigate whether cyclic chemoimmunotherapy has therapeutic efficacy against cancer.

**Methods:** We examined the efficacy of cyclic chemoimmunotherapy with cyclophosphamide and adoptively transferred effector T cells against 5-day, established MCA205 murine skin sarcomas.

**Results:** Cyclophosphamide administration followed by adoptive immunotherapy augmented the trafficking of effector T cells into established tumors. Further, multiple cyclophosphamide administrations helped effector T cells to persist at the sites. Chemoimmunotherapy achieved complete tumor regression even with the transfer of a limited number of effector T cells ( $5 \times 10^6$ ).

**Conclusion:** Cyclic chemoimmunotherapy, which maintains adoptively transferred T cells by impairing regulatory T cells, is a potentially suitable treatment for established tumors.

**Keywords:** Tumor immunotherapy, Cyclophosphamide, Regulatory T cell

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### Introduction

Tumor cells express unique antigens that can be recognized by the host immune system.<sup>1, 2</sup> However, the immune system is usually unable to eliminate proliferating tumor cells, and the host succumbs to the disease.

The reasons for tumor escape have been discussed in recent decades. Tumor cells express a number of molecules that suppress host immune responses.<sup>3</sup> Although antitumor effector T cells are generated in the host, the concomitant, tumor-induced

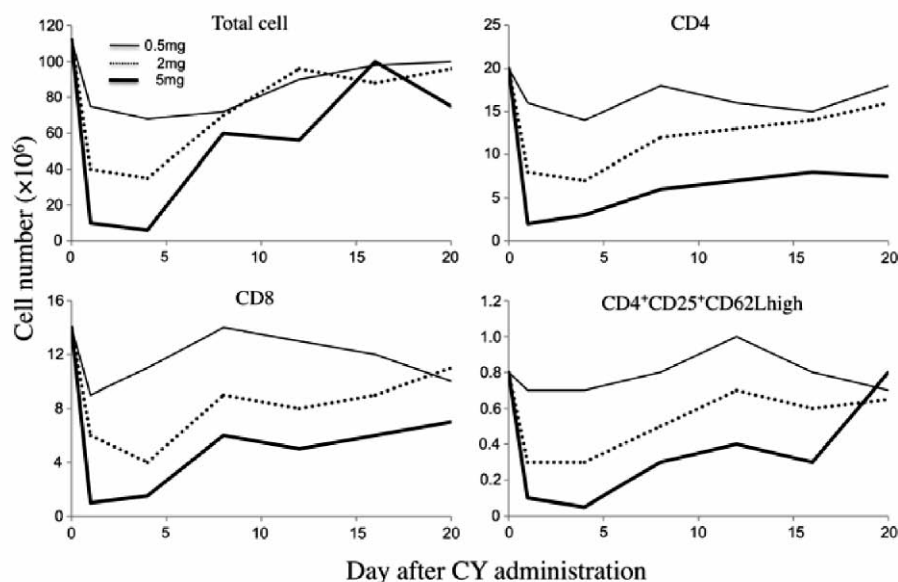
immunosuppressive environment restricts their activities.

Regulatory T cells (Tregs) positive for CD4, CD25 and forkhead box P3 (Foxp3) are considered one of the major factors for tumor-induced immunosuppression. An increased number of Tregs reportedly exist at the local tumor site and in the peripheral blood of patients with various types of cancer.<sup>4-8</sup> Although the precise mechanisms of this increase during tumor progression are still under investigation, tumor-derived factors such as TGF- $\beta$  may play an important role.<sup>9</sup> We have demonstrated previously that Tregs and effector T cells are induced concomitantly in the same tumor-draining lymph nodes (TDLNs), but with different kinetics.<sup>10</sup> The balance between these cell types influences the therapeutic efficacy of adoptive immunotherapy (AIT).

Depletion of Tregs can augment the antitumor immune response and efficacy of antitumor immunotherapy, including dendritic cell-based vaccination.<sup>11-14</sup> Various strategies reportedly control Tregs, such as their depletion with anti-CD25 monoclonal antibody (mAb).<sup>15-17</sup> This mAb is used clinically in organ transplantation to mimic

graft-versus-host disease and is now being investigated for tumor immunotherapy. This strategy, however, depletes activated effector T cells as well, because they too express CD25. Meanwhile, conditioning chemotherapy with cyclophosphamide (CY), a nitrogen mustard cytotoxic agent, reportedly augments the antitumor immune responses in the host.<sup>18</sup> A key mechanism for such immunomodulation is the transient suppression of Tregs.<sup>19</sup> Clinical studies of combined chemoimmunotherapy against melanoma have reported hopeful results.<sup>20-22</sup> In these studies, a single treatment schedule achieved a satisfactory clinical response in some patients. The clinical application of this strategy is being investigated intensively in the treatment of various types of cancer.<sup>23, 24</sup>

We hypothesize that cyclic chemoimmunotherapy is a better strategy to achieve the maximal therapeutic efficacy against cancer. To test our hypothesis, we examined the effect of CY administration on tumor-induced Tregs, and conducted cyclic chemoimmunotherapy with CY and adoptively transferred effector T cells against established murine skin tumors.



**Figure 1.** Effect of cyclophosphamide on the cell number in spleens of naïve mice. Different doses of cyclophosphamide (0.5 mg, thin line; 2.0 mg, dotted line; 5.0 mg, thick line) were administered intraperitoneally on day 0. Spleens were harvested for flow cytometry analysis. The number of splenocytes was counted and multiplied by the fractional percentage of CD4, CD8, and CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> cells. Data are presented as the mean of three mice.

## Materials and Methods

### Mice and tumor cells

Female C57BL/6J (B6) mice were purchased from CLEA Laboratory (Tokyo, Japan). All mice were maintained in a specific pathogen-free environment and cared for in accordance with the institutional guidelines for animal welfare. The experimental animals were 8–10 weeks of age. All experiments were conducted with the permission of the Niigata University Ethics Committee for Animal Experiments.

Single-cell suspensions of MCA205, a chemically-induced fibrosarcoma of B6 origin,<sup>25</sup> were prepared from solid tumors by enzymatic digestion as described previously.<sup>26</sup> An MCA205 tumor cell line was established in vitro.

### Tumor-draining lymph node cells

B6 mice were inoculated subcutaneously with  $2 \times 10^6$  MCA205 tumor cells in both flanks. Inguinal TDLNs were harvested and single-cell suspensions were prepared mechanically, as described previously.<sup>26</sup>

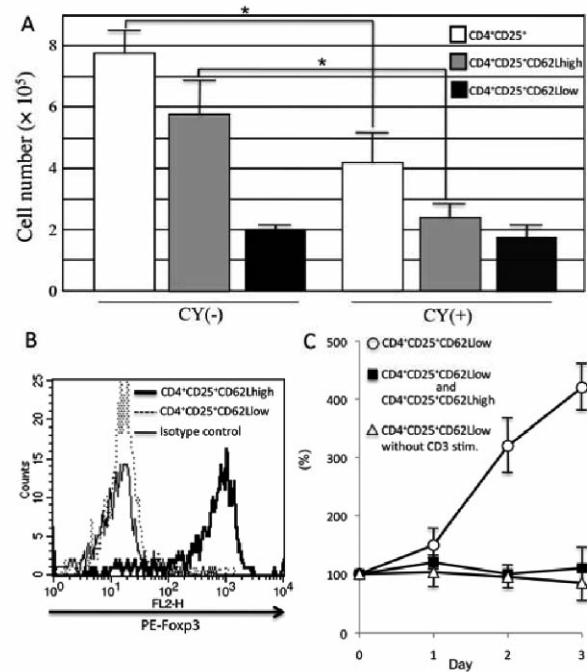
### Fractionation of T cells

T cells in the TDLN cell suspensions were concentrated by passing the suspensions through nylon wool columns (Wako Pure Chemical Industries, Osaka, Japan). For purification of CD4<sup>+</sup>CD25<sup>+</sup> cells, CD4 cells were obtained by using anti-CD4 mAb-coated Dynabeads and Detachabeads (Dyna, Invitrogen, Carlsbad, CA, USA). Then, CD25-positive cells were isolated by using phycoerythrin (PE)-conjugated anti-CD25 mAb and anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Cell purity was greater than 95%. T cells with down-regulated CD62L expression (CD62L<sup>low</sup>) in the TDLN were isolated by a panning technique, using T-25 flasks precoated with goat anti-rat Ig Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and anti-CD62L mAb (MEL14). To yield highly purified (>90%) CD62L<sup>low</sup> cells, the non-adherent cells were further depleted of CD62L-positive cells using anti-CD62L mAb-

coated Dynabeads M-450 (Dyna, Invitrogen). T cells with high CD62L expression (CD62L<sup>high</sup>) were obtained by scraping the adherent cells with a rubber policeman.

### Proliferation assay

T cells isolated from TDLNs were stimulated with immobilized anti-CD3 mAb for 48 h in 2 mL of complete medium (CM) on 24-well plates at  $2 \times 10^6$  cells/mL. Complete medium consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids, 1  $\mu$ mol/L sodium pyruvate, 2 mmol/L fresh L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate (all



**Figure 2.** Effect of cyclophosphamide on the number of regulatory T cells in tumor-draining lymph nodes. Six mice were inoculated subcutaneously with  $2 \times 10^6$  MCA205 tumor cells in both flanks. Cyclophosphamide was administered to 3 mice 1 day before and 6 days after tumor inoculation. CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>low</sup> cell fractions were purified from CD4<sup>+</sup>CD25<sup>+</sup> T cells harvested from day-12 tumor-draining lymph node cells. Results are expressed as the mean cell number per lymph node  $\pm$  SD. Statistical analysis was done with Student's *t*-test. \*,  $P < 0.01$ . (A)

Flow cytometry analysis of Foxp3 expression on CD4<sup>+</sup>CD25<sup>+</sup> T cells. (B) T cell proliferation in the presence of 10 U/mL recombinant interleukin 2 (rIL-2) after stimulation with anti-CD3 monoclonal antibody. The ratio of CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>low</sup> cells to CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> cells was 2:1. Three wells were analyzed for each condition. (C)

from Life Technologies, Grand Island, NY, USA), and  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). CD62L<sup>low</sup> T cells were labeled with 5  $\mu$ M of the intracellular green fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen) in Hank's balanced salt solution (HBSS) at 37 °C for 15 min and washed twice before CD3 stimulation. The ratio of CD62L<sup>low</sup> T cells to CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> T cells was 2:1. After stimulation for 48 h, the cells were counted, washed twice with HBSS and cultured in CM supplemented with 10 U/mL human recombinant interleukin 2 (rIL-2) at  $1 \times 10^5$  cells/mL. Three wells were analyzed for each condition.

### Adoptive immunotherapy

To establish skin tumors, mice were inoculated subcutaneously with  $1 \times 10^6$  MCA205 tumor cells suspended in 100  $\mu$ L of HBSS. Five days later, effector T cells were transferred intravenously to the tumor-bearing mice. The effector T cells were generated from 12-day TDLN cells. CD62L<sup>low</sup>

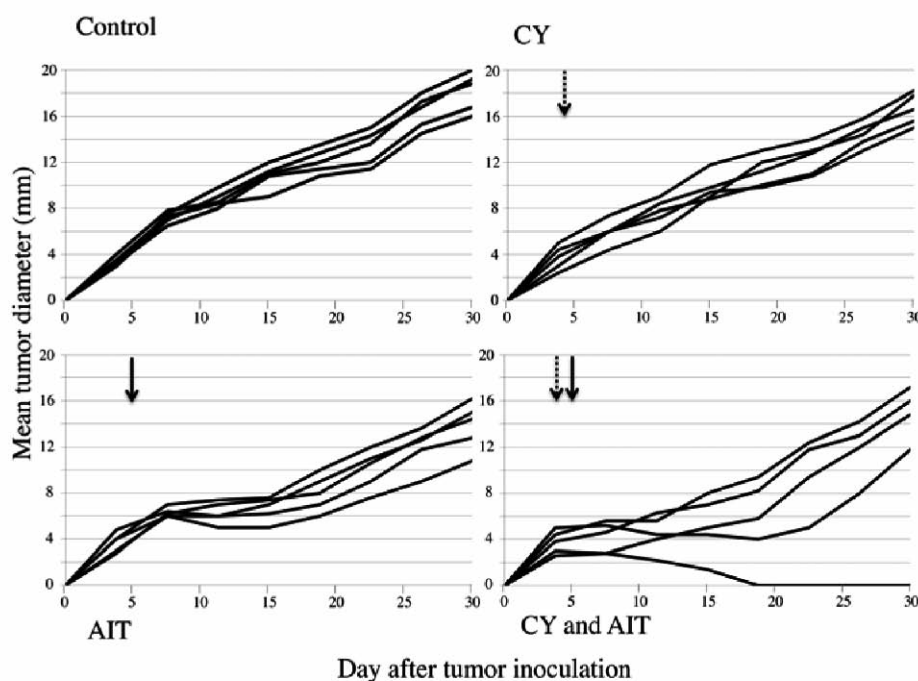
T cells in the TDLNs were purified, activated by immobilized anti-CD3 mAb (2C11) for 2 days, further cultured with 20 U/mL of rIL-2 in CM for 3 days and used as effector T cells. The effector T cells were prelabeled with CFSE for tracking in vivo. In some experiments, CY was administered intraperitoneally to the tumor-bearing mice (2 mg/mouse). The treatments were repeated weekly and perpendicular diameters of the skin tumors were monitored.

### Flow cytometry analysis

PE-conjugated anti-Foxp3 mAb was purchased from BD Bioscience (San Jose, CA, USA). Foxp3 expression on purified CD4<sup>+</sup>CD25<sup>+</sup> cells was analyzed by using a fluorescence-activated cell sorter scan flow microfluorometer (BD Bioscience).

### Statistical analysis

The results were expressed as mean values  $\pm$  SD. Two-sided Student's t-tests were used for all comparisons.  $P < 0.01$  was considered significant.



**Figure 3.** Changes in the mean diameter of 5-day established MCA205 skin tumors after cyclic chemoimmunotherapy. Twenty million effector T cells generated from tumor-draining lymph nodes were adoptively transferred to the tumor-bearing mice. Cyclophosphamide was administered 1 day before the transfer. The tumor diameters were measured twice weekly with calipers, and their size was recorded as the average of 2 perpendicular diameters. Each group contained 5 mice. The dotted arrows indicate cyclophosphamide administration and solid arrows indicate adoptive immunotherapy. The data are representative of 3 independent experiments with similar results.



## Results

### *Efficacy of cyclophosphamide administration in naïve mice*

Cyclophosphamide has been shown to selectively suppress Tregs.<sup>18, 19</sup> We demonstrated previously that CD4<sup>+</sup>CD25<sup>+</sup> CD62L<sup>high</sup> cells represented Tregs and CD4<sup>+</sup>CD25<sup>+</sup> CD62L<sup>low</sup> T cells represented CD4 effector T cells.<sup>10</sup> Previous reports indicated that 50~150 mg/kg (approx. 1~3 mg/mouse) of CY is an appropriate dose to suppress Tregs in rodents.<sup>27, 28</sup> We titrated the dose of CY in naïve mice. Cyclophosphamide administration resulted in transient suppression of CD4, CD8 and CD4<sup>+</sup>CD25<sup>+</sup> CD62L<sup>high</sup> T cells (Treg) in spleens in a dose-dependent manner (Figure 1). Regulatory T cells decreased transiently from day 2 to day 4 of treatment and then recovered gradually. The administration of 5 mg

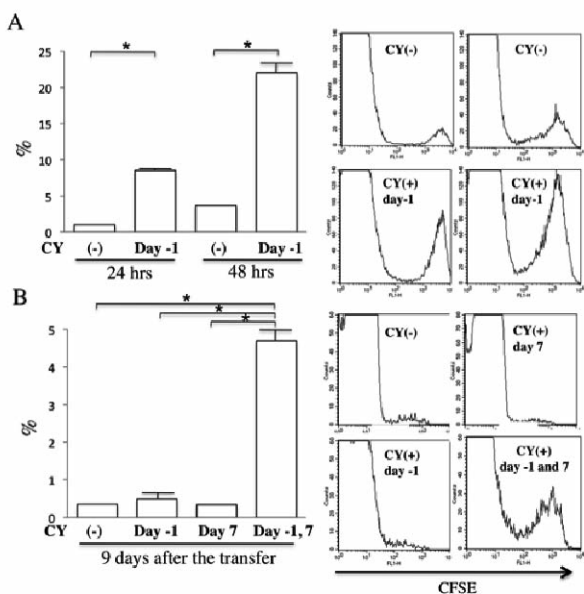
CY resulted in the greatest suppression; however, it induced 20% lethality in treated mice. Therefore, we chose 2 mg CY for the following experiments.

### *Cyclophosphamide selectively depleted regulatory T cells in tumor-bearing mice*

We demonstrated previously that 12-day TDLN cells are an excellent source of effector T cells for AIT. We examined the effect of CY on the regulatory–effector T cell balance in TDLNs. Because of the transient effect of CY, we administered 2 mg CY before tumor inoculation (day 1) and during tumor growth (day 6), and harvested TDLN cells on day 12. The treatment did not affect tumor growth; however, fluorescence-activated cell sorter analysis showed that the number of CD4<sup>+</sup>CD25<sup>+</sup> CD62L<sup>high</sup> T cells decreased significantly (Figure 2A). In contrast, CD4<sup>+</sup>CD25<sup>+</sup> CD62L<sup>low</sup> T cells were conserved in the TDLNs even with CY administrations. A single CY administration on day 1 or day 6 had little impact on the 12-day TDLNs (data not shown). CD4<sup>+</sup>CD25<sup>+</sup> CD62L<sup>high</sup> T cells in the TDLNs expressed Foxp3 and suppressed proliferation of effector T cells upon stimulation with anti-CD3 mAb and rIL-2 (Figures 2B, C). Therefore, CY administrations selectively impaired Tregs in the TDLNs during tumor progression.

### *Cyclophosphamide augmented the efficacy of adoptive immunotherapy*

Regulatory T cells increase in number during tumor progression, and might impair the transferred effector T cells. For combined AIT and CY administration, we adoptively transferred effector T cells generated from the TDLNs 1 day after CY administration. We demonstrated previously that 3-day MCA205 skin tumors are curable by AIT with 2×10<sup>6</sup> effector T cells, following sublethal whole-body irradiation;<sup>10</sup> however, once the tumors had grown for 5 days, they were not responsive to the same strategy (data not shown). Therefore, we used 5-day skin tumors as the treatment model in the present study. As shown in Figure 3, combined AIT and



**Figure 4.** Accumulation of transferred effector T cells in the tumors. Carboxyfluorescein succinimidyl ester-labeled effector T cells ( $30 \times 10^6$ ) were transferred to mice. MCA205 tumor cells ( $6 \times 10^6$ ) were inoculated into the flanks bilaterally 5 days before the transfer. Cyclophosphamide (2 mg) was administered 1 day before and/or 7 days after the transfer. The tumors were resected and digested enzymatically 24 h, 48 h (A), and 9 days (B) later for flow cytometry analysis. Dead cells were excluded by propidium iodide staining before the analysis. Bar graphs indicate the percentage of effector T cells (carboxyfluorescein succinimidyl ester-labeled effector T-positive cells) among the digested cells from 3 independent experiments. Results are expressed as mean values  $\pm$  SD. Statistical analysis was done with Student's t-test. \*,  $P < 0.01$ . Histograms show representative flow cytometry data.

CY administration suppressed tumor growth. However, complete tumor regression was not achievable despite an increased number (up to  $20 \times 10^6$ ) of transferred effector T cells.

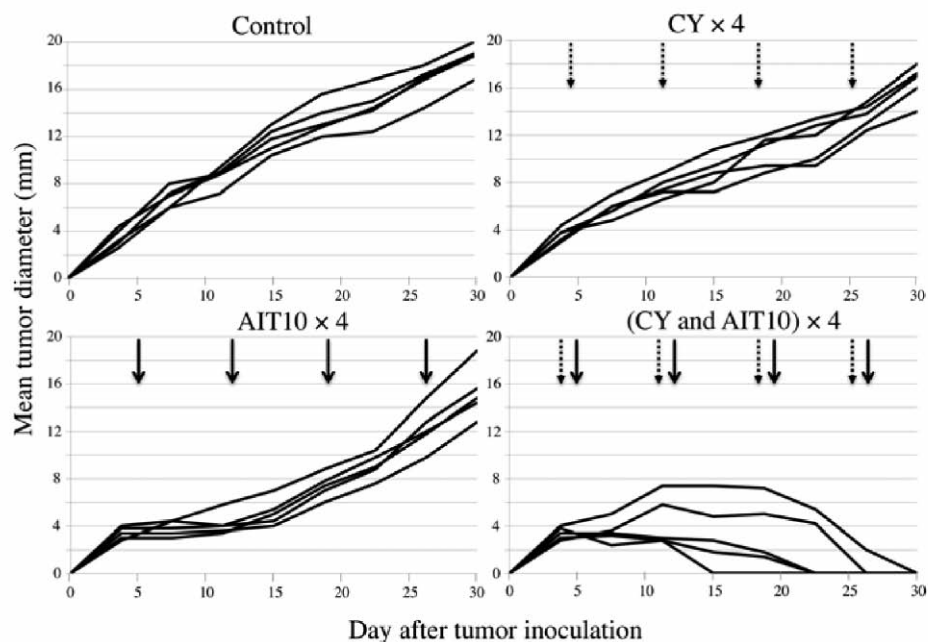
### *Cyclophosphamide maintained adoptively transferred effector T cells in vivo*

We analyzed whether CY treatment affects adoptively transferred effector T cells. Carboxy-fluorescein succinimidyl ester-labeled effector T cells were transferred to naïve mice and mice bearing 5-day established skin tumors. Cyclophosphamide was administered 1 day before the adoptive transfer. Tumor masses were excised and digested enzymatically for flow cytometry analysis. As shown in Figure 4A, CY treatment enhanced the accumulation of adoptively transferred effector T cells in tumors. To examine the efficacy of repeated chemotherapy on the fate of infused effector T cells, we analyzed their accumulation in the tumors after repeated CY treatments. Cyclophosphamide was administered 1 day before, or 7 days after the adoptive transfer of effector T cells, or on both of these days. As

shown in Figure 4B, few effector T cells were detected on day 9 after a single CY administration. In contrast, and interestingly, the effector T cells persisted in the tumors even 9 days after the adoptive transfer when CY was administered twice.

### *Cyclic chemoimmunotherapy against established skin tumors*

The data illustrated in Figure 4 prompted us to examine the therapeutic efficacy of cyclic chemoimmunotherapy. We repeated AIT following weekly CY administration. As shown in Figure 5, cyclic chemoimmunotherapy achieved complete regression of 5-day established skin tumors; in contrast, cyclic AIT or cyclic CY administration alone did not. We estimated the number of effector T cells responsible for tumor regression. Cyclic CY administration enabled the transferred effector T cells to persist in the tumors for a longer time (Figure 4B). However, single AIT after cyclic CY administration did not achieve tumor regression, even with an increased number (up to  $40 \times 10^6$ ) of transferred effector T cells (Figure 6). In contrast,



**Figure 5.** Cyclic chemoimmunotherapy for 5-day established MCA205 skin tumors. MCA205 tumor cells ( $1 \times 10^6$ ) were injected into mice. Cyclophosphamide was administered weekly from day 4 of tumor growth. Ten million effector T cells were adoptively transferred (AIT10) weekly from day 5 of tumor growth. The tumor diameter was measured by calipers twice a week. The dotted arrows indicate cyclophosphamide and solid arrows indicate adoptive transfer. Data are representative of 3 independent experiments with similar results.

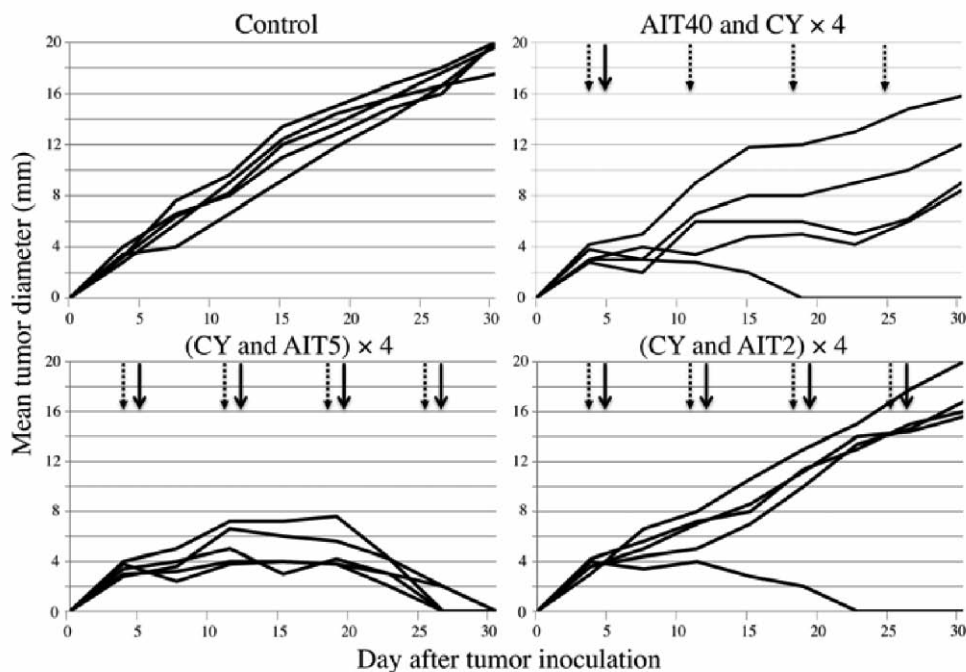
when the treatment was administered repeatedly, as few as 5 million effector T cells per adoptive transfer had therapeutic efficacy. These data suggest that cyclic chemoimmunotherapy was effective in treating the established tumors.

## Discussion

In this study, we demonstrated that cyclic chemoimmunotherapy with CY administration after AIT achieved complete regression of established murine skin tumors, which were not treatable with single chemoimmunotherapy. Adoptive immunotherapy is an attractive strategy with a long history. Direct infusion of effector T cells capable of eradicating tumor cells has succeeded in various animal models.<sup>29-31</sup> However, its efficacy in the clinic has been equivocal.<sup>32, 33</sup> One of the key factors for successful AIT is the mitigation of tumor-induced immunosuppression. With the Response Evaluation Criteria in Solid Tumors (RECIST), Dudley et al. found that the combined modality of AIT and nonmyeloablative chemotherapy for advanced melanoma achieved a remarkable reduction in tumor volume.<sup>20-22</sup>

These reports had a great impact on the field, because previous clinical studies concerning tumor immunotherapy did not demonstrate such therapeutic efficacy against advanced metastatic disease. Most importantly, it became apparent that nonmyeloablative chemotherapy with CY and fludarabine could overcome tumor-induced immunosuppression.

Chemotherapeutic agents such as CY have immune-potentiating effects against tumors. Recent studies have revealed that CY affects both cellular and humoral immune responses.<sup>34,35</sup> Among the various mechanisms, the suppression of Tregs by CY is essential, because Tregs increase in number in tumor-bearing hosts and play a pivotal role in tumor-induced immunosuppression.<sup>19</sup> We previously demonstrated that Tregs and effector T cells with antitumor efficacy are induced concomitantly in TDLNs, but with different kinetics, and that CD62L is a useful marker for discriminating between these cells.<sup>10</sup> In the present study, CY administration selectively suppressed Tregs in naive spleens as well as TDLNs (Figures 1 and 2). The mechanisms



**Figure 6.** Estimated numbers of effector T cells required for the therapeutic efficacy of cyclic chemoimmunotherapy. The experimental design was similar to that described in Figure 5, but  $40 \times 10^6$  (AIT40),  $5 \times 10^6$  (AIT5) or  $2 \times 10^6$  (AIT2) effector T cells was transferred. The dotted arrows indicate cyclophosphamide administration, whereas solid arrows indicate adoptive transfer. Each group contained 5 mice. The data are representative of 3 independent experiments with similar results.

underlying this selective depletion are not clear. Recently, by using Tregs from healthy volunteers, Zhao et al. demonstrated that selective depletion of Tregs by CY is associated with reduced intracellular ATP levels.<sup>36</sup>

We demonstrated that CY administration followed by AIT retards tumor growth. However, complete regression of 5-day established skin tumors was not achievable with single chemoimmunotherapy (Figure 2). The lifetime of effector T cells after adoptive transfer has been reported to be shorter than expected.<sup>37</sup> Terminally activated effector T cells that exhibit strong antitumor efficacy in vitro show impaired survival in vivo. Therefore, the maintenance of effector T cells after their transfer is critical in augmenting the therapeutic efficacy of AIT.<sup>38,39</sup> Our data showed that the adoptively transferred effector T cells were maintained in regional tumor tissue for a longer time with the administration of CY (Figure 4A). Wada et al. have reported that low-dose metronomic CY controlled Tregs.<sup>40</sup> Repeated CY administrations might suppress reemerging Tregs that may interfere with transferred effector T cells. Interestingly, our data showed that effector T cells were maintained for as long as 9 days after the adoptive transfer when CY was administered both before and after the transfer (Figure 4B). Markasz et al. reported that CY enhanced the antitumor effect of effector T cells in vitro.<sup>41</sup> Nonetheless, our data showed that a single adoptive transfer of  $40 \times 10^6$  effector T cells with multiple CY administrations was less effective than cyclic chemoimmunotherapy with fewer effector T cells ( $5 \times 10^6$ ) per transfer (Figures 5 and 6). Each growth curve suggests a transient efficacy for each chemoimmunotherapy. It should be emphasized that the attack on established tumors in waves by the transfer of divided doses of effector T cells following chemotherapy may achieve complete tumor regression.

For cyclic AIT to be successful in the clinic, it is essential to prepare a sufficient number of effector T cells with strong antitumor activities. Tumor-draining lymph node cells and tumor-infiltrating lymphocytes are excellent sources

because they are already sensitized to tumor-associated antigens.<sup>26</sup> However, there are practical limitations in harvesting such cells repeatedly from patients.<sup>42</sup> It is still difficult to generate good effector T cells from peripheral blood mononuclear cells to be used for treating cancer. Therefore, the limited materials should be used in the best way possible. Our data support the concept of fractionated transfers of a limited amount of effector T cells combined with CY chemotherapy.

In this study, the therapeutic efficacy of cyclic chemoimmunotherapy depended on the number of effector T cells (Figure 6). Therefore, the quality of effector T cells is another important aspect. In vitro assays to evaluate effector T-cell functions such as cytotoxicity are believed to be the most reliable predictors of therapeutic efficacy. In fact, less mature T cells have been shown to exhibit better efficacy than terminally activated effector T cells.<sup>37</sup> We have previously demonstrated the successful generation of antitumor effector T cells from naïve T cells by coculture with dendritic-tumor fusion cells,<sup>43</sup> and Plautz et al. reported a long-term megaculture method for generating effector T cells from TDLNs.<sup>41-44</sup> The genetic modification of effector T cells may be another option for generating viable effector T cells.<sup>42,45,46</sup> These developing technologies for producing a stable supply of effector T cells hold the potential to enable the clinical application of cyclic chemoimmunotherapy.

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