

Different mechanisms for anti-tumor effects of low- and high-dose cyclophosphamide

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Abstract. It is known that, besides its direct cytotoxic effect as an alkylating chemotherapeutic agent, cyclophosphamide also has immuno-modulatory effects, such as depletion of CD4⁺CD25⁺ regulatory T cells. However, its optimal concentration has not yet been fully elucidated. Therefore, we first compared the effects of different doses of cyclophosphamide on T cell subsets including CD4⁺CD25⁺ T cells in mice. Cyclophosphamide (20 mg/kg) decreased the numbers of splenocytes, CD4⁺ and CD8⁺ T cells by ~50%, while a decline in CD4⁺CD25⁺ T cell number was more profound, leading to the remarkably lower ratios of CD4⁺CD25⁺ T cells to CD4⁺ T cells. In contrast, 200 mg/kg cyclophosphamide severely decreased the numbers of all the T cell subsets by >90% although the decreased ratios of CD4⁺CD25⁺ T cells to CD4⁺ T cells were still observed. Next, low-dose cyclophosphamide significantly inhibited *in vivo* growth of murine hepatoma MH129 tumor in immuno-competent but not immuno-deficient mice. This anti-tumor effect was abolished by CD4⁺CD25⁺ T cell repletion. In contrast, high-dose cyclophosphamide exhibited similar anti-tumor effects in both mice. In addition, contrary to antibody-mediated CD4⁺CD25⁺ T cell depletion, administration of low-dose cyclophosphamide after tumor inoculation was more efficacious than the prior administration. Our data show that low-dose cyclophosphamide selectively depletes CD4⁺CD25⁺ T cells, leading to enhanced anti-tumor effects against pre-existing tumors, while the anti-tumor effect of high-dose cyclophosphamide is solely attributed to its direct cytotoxicity. These findings

appear to be highly crucial in a clinical setting of combined chemotherapy and immunotherapy for cancer treatment.

Introduction

Chemotherapy and immunotherapy are generally regarded as unrelated or even mutually exclusive in cancer treatment, because chemotherapy kills not only target cancer cells but also immune cells, inducing systemic immune suppression and dampening the therapeutic efficacy of immunotherapy. In this regard, however, cyclophosphamide appears an exceptional chemotherapeutic agent (1,2). Besides its direct cytotoxic effect as an alkylating agent, cyclophosphamide is reported to modulate the immune system in hosts (1,2). Examples for this include (i) enhancement of dendritic cell-based anti-tumor immunity by increased tumor antigens released from tumor cells dying of cyclophosphamide-induced apoptosis (3), (ii) increased type-I interferon production and evolution of CD44^{hi} memory T cell response by cyclophosphamide (4), (iii) induction of homeostatic T cell proliferation by cyclophosphamide-mediated lymphopenia that enhances some cancer vaccines (5,6), and (iv) down-regulation of T-cell derived IL-10 and TGF- β productions by cyclophosphamide (7). More importantly, recent studies show selective suppression by cyclophosphamide of CD4⁺CD25⁺ naturally occurring regulatory T cells (8-11), which are widely believed to play a key role in immune tolerance (12). Although it is widely believed that 'low-dose' cyclophosphamide augments the immune response, the optimal concentration has not yet been fully elucidated. Thus, the amounts of cyclophosphamide used vary from 10 to 300 mg/kg in studies on immuno-potential of low-dose cyclophosphamide (2) and from 30 to 200 mg/kg in those on cyclophosphamide-mediated CD4⁺CD25⁺ T cell suppression (8-11). In this article, therefore, we compared the effects of different doses of cyclophosphamide on T cell subsets including CD4⁺CD25⁺ T cells and also on tumor immunity in mice. Our results clearly demonstrate that low-dose (20 mg/kg), but not high-dose (200 mg/kg), cyclophosphamide selectively suppresses the number of CD4⁺CD25⁺ T cells but spares those of conventional CD4⁺ and CD8⁺ T cells in spleen, and efficiently inhibits, through CD4⁺CD25⁺ T cell

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Key words: cyclophosphamide, CD4⁺CD25⁺ naturally occurring regulatory T cells, tumor immunity, MH129 hepatoma cells, effector T cells

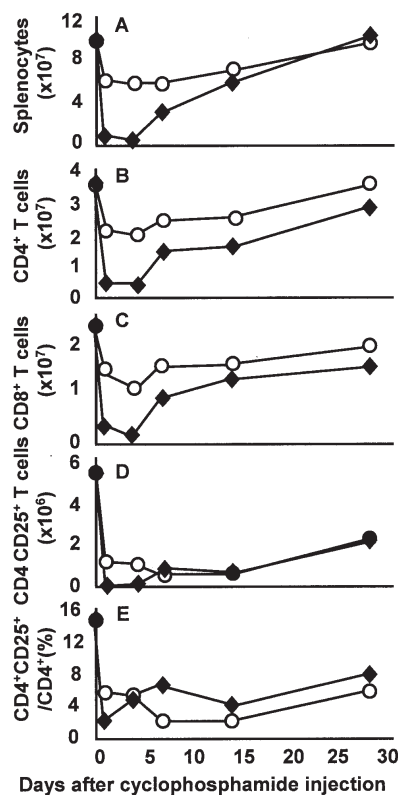


Figure 1. Alterations in T cell subsets following intraperitoneal injection of low- or high-dose cyclophosphamide. After 1,4,7,14 and 28 days after intraperitoneal injection of 20 or 200 mg/kg cyclophosphamide, numbers of splenocytes, CD4⁺, CD8⁺ and CD4⁺CD25⁺ T cells were determined as described in the Materials and methods. The data are means of two mice in each group. ○ and ●, 20 and 200 mg/kg cyclophosphamide, respectively. The same experiments were repeated at least twice with similar results.

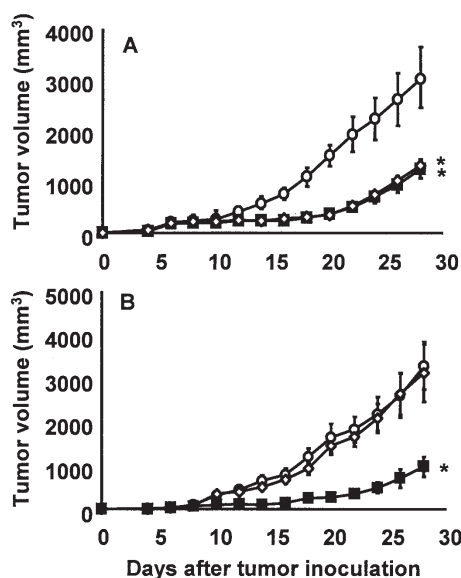


Figure 2. Anti-tumor effects of low- and high-dose cyclophosphamide in MH129 tumor-bearing C3H/HeN and nude mice. Mice (A, C3H/HeN; B, BALB/c nu/nu) were inoculated with 5×10^5 MH129 cells on day 0. Groups of mice were also treated with 20 or 200 mg/kg cyclophosphamide on day 7, and injected with 4×10^6 CD4⁺CD25⁺ T cells on day 8. Tumor sizes were monitored for 4 weeks. ○, control mice; ◇, mice treated with 20 mg/kg cyclophosphamide; ■, mice treated with 200 mg/kg cyclophosphamide; ▲, mice treated with 20 mg/kg cyclophosphamide and 4×10^6 CD4⁺CD25⁺ T cells. The data are means \pm SEM (n=8). * $p < 0.05$, versus control mice (t-test). The same experiments were repeated at least twice with similar results.

depletion, *in vivo* growth of pre-existing murine hepatoma MH129 tumor. These findings appear to be highly crucial in a clinical setting of combined chemotherapy and immunotherapy for cancer treatment.

Materials and methods

Cell lines and mice used. MH129 cells, a mouse hepatoma cell line (13), were maintained in RPMI-1640 medium with 10% fetal calf serum and antibiotics. Six-week-old female C3H/HeN and BALB/c nu/nu mice were purchased from Charles River Japan (Tokyo, Japan) and kept in a specific pathogen-free facility. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals of Nagasaki University.

Studies on the effect of cyclophosphamide on T cell subsets in the spleen. C3H/HeN mice were intraperitoneally injected with cyclophosphamide (20 or 200 mg/kg; Sigma, St. Louis, MI), and numbers of splenocytes, CD4⁺, CD8⁺ and CD4⁺CD25⁺ T cells were monitored for up to 4 weeks. The numbers of splenocytes were counted using a hemocytometer after lysis of red blood cells with ammonium chloride. The numbers of CD4⁺, CD8⁺ and CD4⁺CD25⁺ T cells were determined with FITC-conjugated anti-mouse CD4 (H129.19), PE-conjugated anti-CD8 (53-6.7) and PE-conjugated anti-CD25 (7D4) (PharMingen, San Diego, CA), respectively, on a FACScan flow cytometre using CellQuest software (BD Biosciences, Mountain View, CA).

Studies on the effect of low-dose cyclophosphamide on *in vivo* MH129 tumor growth. MH129 cells (5×10^5 cells/mouse) were subcutaneously injected into the flanks of mice. Tumor sizes were determined from caliper measurement using the standard formula (length \times width²/2). Groups of mice were treated by either intraperitoneal injection of cyclophosphamide (20 or 200 mg/kg) (Sigma) or 500 μ g/mouse anti-CD25 antibody before or after tumor cell inoculation. A group of mice were also intraperitoneally injected with 4×10^6 cells purified CD4⁺CD25⁺ T cells at the indicated time point. Anti-CD25 monoclonal antibody was purified from ascites of mice intraperitoneally injected with hybridoma PC61 using HiTrapTM protein G HP column (Amersham, Piscataway, NJ). CD4⁺CD25⁺ T cells (>90% pure) were isolated from splenocytes of naïve mice using a SpinSep Murine CD4⁺ T-Cell kit (Veritas, Tokyo, Japan) and MACS CD25 MicroBead kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Confirmation of CD25⁺ cell depletion by PC61 was determined by staining splenocytes 4 days after antibody treatment with anti-CD25 antibody that recognize a different epitope of CD25 (7D4).

Histology. Tumor histology was examined on formalin-fixed tissue sections stained with hematoxylin and eosin (H&E).

Results

Low-, but not high-dose cyclophosphamide selectively depletes CD4⁺CD25⁺ T cells. We first examined the effects of low- and high-dose cyclophosphamide on the kinetics of T

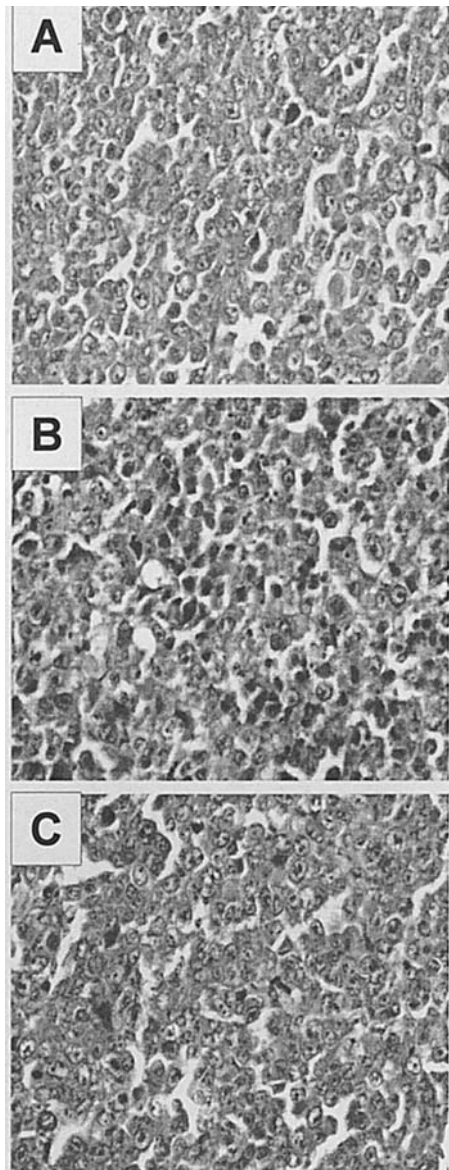


Figure 3. H&E staining of MH129 tumors from control mice (A) and mice treated with 20 or 200 mg/kg cyclophosphamide (B and C). Tumors were removed on day 22 from mice. Magnification of x400.

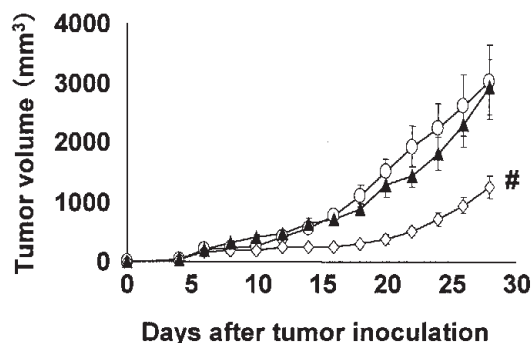


Figure 4. Anti-tumor effects of low-dose cyclophosphamide with/without repletion of CD4⁺CD25⁺ T cells in MH129 tumor-bearing C3H/HeN mice. Mice were inoculated with 5×10^5 MH129 cells on day 0. Groups of mice were also treated with 20 mg/kg cyclophosphamide on day 7, and injected with 4×10^6 CD4⁺CD25⁺ T cells on day 8. Tumor sizes were monitored for 4 weeks. \circ , control mice; \diamond , mice treated with 20 mg/kg cyclophosphamide; \blacktriangle , mice treated with 20 mg/kg cyclophosphamide and 4×10^6 CD4⁺CD25⁺ T cells. The data are means \pm SEM (n=8). *p<0.05, versus control mice (t-test). The same experiments were repeated at least twice with similar results.

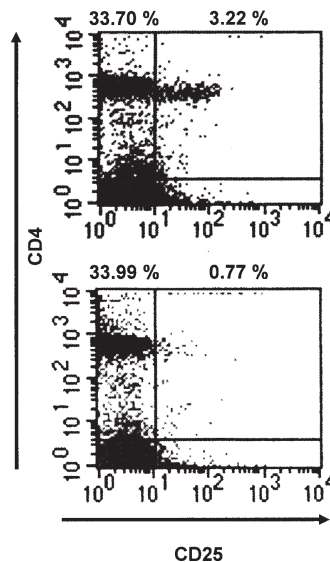


Figure 5. Flow cytometric analysis of CD4 and CD25 expression on splenocytes in mice untreated (A) or treated with 0.5 mg anti-CD25 antibody (B). Four days after antibody treatment, splenocytes were analyzed for CD4 and CD25 expressions by FACSscan as described in Materials and methods.

lymphocyte subsets in the spleens of C3H/HeN mice. Flow cytometric analysis of splenocytes was performed 1, 4, 7, 14 and 28 days after intraperitoneally injecting 20 or 200 mg/kg cyclophosphamide (day 0). Fig. 1 shows that 20 mg/kg cyclophosphamide decreased the numbers of splenocytes, CD4⁺ and CD8⁺ T cells by ~50% from day 1. The decrease peaked on day 4 and continued for at least 2 weeks. However, a decline in CD4⁺CD25⁺ T cell number was more profound (~85% decrease) and recovered more slowly than CD4⁺ cells, thus leading to the remarkably lower ratios of CD4⁺CD25⁺ T cells to CD4⁺ T cells throughout the experimental period. In contrast, 200 mg/kg cyclophosphamide severely decreased the numbers of all the T cell subsets examined by >90% although the decreased ratios of CD4⁺CD25⁺ T cells to CD4⁺ T cells were still observed. These data clearly indicate the selective suppression of CD4⁺CD25⁺ regulatory T cells by low-dose, but not high-dose, cyclophosphamide.

Anti-tumor effect of low-dose cyclophosphamide is immune-mediated, while that of high-dose cyclophosphamide is attributed solely to direct cytotoxic effect. To see how critically the aforementioned phenomenon plays a role in tumor immunity, anti-tumor effects of low- and high-dose cyclophosphamide were compared in an *in vivo* tumor model with a mouse hepatoma cell line, MH129, and syngeneic immuno-competent C3H/HeN and immuno-incompetent nude mice. Surprisingly, both 20 and 200 mg/kg cyclophosphamide injected 7 days after tumor cell inoculation significantly suppressed the growth of MH129 tumors in C3H/HeN mice (Fig. 2A). However, the suppressive effect of low-dose cyclophosphamide was no longer observed in nude mice (Fig. 2B), indicating that the anti-tumor effect of low-dose cyclophosphamide observed in immuno-competent mice appears to be attributed to anti-tumor immunity. In contrast, high-dose cyclophosphamide was equally effective in both mice, indicating that the effect is solely due to direct cytotoxicity.

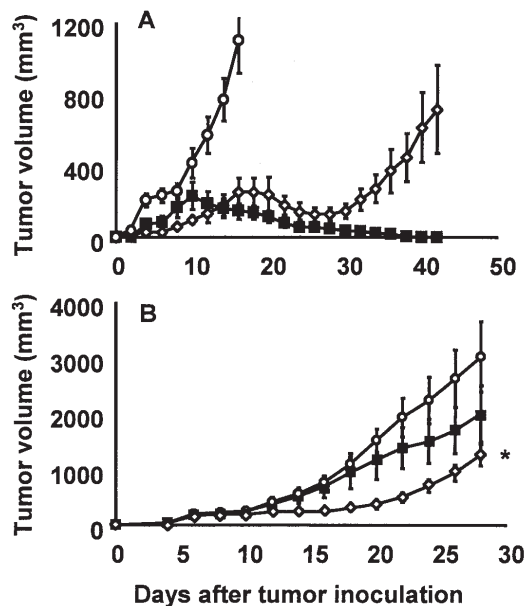


Figure 6. Comparison of anti-tumor effects of low-dose cyclophosphamide and anti-CD25 antibody administered before and after tumor cell inoculation in C3H/HeN mice. C3H/HeN mice were inoculated with 5×10^5 MH129 cells on day 0. Groups of mice were also treated with 500 μ g/mouse anti-CD25 antibody (A) or 20 mg/kg cyclophosphamide (B) on day -4 or +4. Tumor sizes were then monitored for 4 or 7 weeks. \circ , control mice; \diamond , mice treated with cyclophosphamide or anti-CD25 antibody after tumor cell inoculation; \blacksquare , mice treated with cyclophosphamide or anti-CD25 antibody before tumor cells inoculation. The data are means \pm SEM ($n=8$). * $p<0.05$, versus control mice. The same experiments were repeated at least twice with similar results.

In histological examinations (Fig. 3), tumors from mice treated with low-dose cyclophosphamide showed higher intratumoral lymphocyte infiltrations as compared to those in control and high-dose cyclophosphamide-treated mice, data compatible with immune and non-immune mediated anti-tumor effects of low- and high-dose cyclophosphamide, respectively.

Anti-tumor effect of low-dose cyclophosphamide is mediated by depletion of CD4⁺CD25⁺ T cells. The next study was performed to evaluate whether anti-tumor immunity induced by low-dose cyclophosphamide resulted from the selective CD4⁺CD25⁺ T cell depletion. For this purpose, following MH129 inoculation on day 0 and low-dose cyclophosphamide injection on day 7, CD4⁺CD25⁺ T cells (4×10^6 cells/mouse) purified from naïve mice were injected on day 8. Fig. 4 shows that repletion of CD4⁺CD25⁺ T cells completely abolished the anti-tumor effect of low-dose cyclophosphamide. These data clearly implicate the anti-tumor effect of low-dose cyclophosphamide in its selective depletion of CD4⁺CD25⁺ T cells.

Timing of administration to obtain the optimal anti-tumor immunity is different between low-dose cyclophosphamide and anti-CD25 antibody. Because monoclonal anti-CD25 antibody (PC61) has been widely used to deplete CD4⁺CD25⁺ T cells in previous studies (14-16), the consequence of antibody-mediated CD4⁺CD25⁺ T cell depletion was compared to that of cyclophosphamide-mediated CD4⁺CD25⁺ T cell

depletion in the MH129 tumor model. In our preliminary dose-escalating experiment, intraperitoneal injection of 0.5 mg anti-CD25 antibody (PC61) maximally depleted CD4⁺CD25⁺ T cells (Fig. 5). Fig. 6 shows that four day-prior injection of anti-CD25 antibody completely eradicated MH129 tumors but, 4 days later, the injection only transiently inhibited tumor growth, which is consistent with data previously reported with several cell lines including Meth A, MOPC-70A and RL Male1 cells (15,16). However, the results were opposite in the case of cyclophosphamide; injection of cyclophosphamide on day -4 of tumor cell inoculation was less effective than that on day +4.

Discussion

Although cyclophosphamide-induced suppression of CD4⁺CD25⁺ regulatory T cells has previously been demonstrated, attention has not been focused on the concentrations of cyclophosphamide used. Thus, the amounts used varied from 30 to 200 mg/kg in mice and rats (8-11). Furthermore, an immuno-enhancing effect has also been described with doses of cyclophosphamide ranging from 10 to 300 mg/kg (2). Therefore, we first compared the outcomes of high-dose (200 mg/kg) and low-dose (20 mg/kg) cyclophosphamide administration on T cell subsets in mice. Our data clearly demonstrate that, although both high- and low-dose cyclophosphamide markedly suppressed the number of CD4⁺CD25⁺ T cells and the ratios of CD4⁺CD25⁺ T cells to CD4⁺ T cells, their effects on the numbers of CD4⁺ T and CD8⁺ T cells were quite different. Thus, decreases in absolute numbers of CD4⁺ T and CD8⁺ T cells by high-dose cyclophosphamide were much more evident than those by low-dose cyclophosphamide (>90% versus ~50% decreases). These observations indicate that CD4⁺CD25⁺ regulatory T cells are selectively suppressed, but conventional, effector T cells appear to be spared by low-dose cyclophosphamide. In contrast, high-dose cyclophosphamide induces quantitatively substantial decreases in all T cell subsets, presumably eradicating both regulatory and effector T cells.

Regarding the mechanism(s) for this selective depletion of CD4⁺CD25⁺ T cells with cyclophosphamide, Lutsiak *et al* (10) showed the increased sensitivity of CD4⁺CD25⁺ T cells to apoptosis in 100 mg/kg cyclophosphamide-treated mice, leading to the impaired homeostatic proliferation of this T cell subpopulation. Ercolini *et al* (11) also demonstrated selective depletion of the cycling population of CD4⁺CD25⁺ T cells with 100 mg/kg cyclophosphamide. In addition, Ikezawa *et al* (9) and Lutsiak *et al* (10) found that cyclophosphamide suppressed not only number but also functional property of CD4⁺CD25⁺ T cells.

The long-lasting depletion of CD4⁺CD25⁺ T cells by low-dose cyclophosphamide enabled us to observe the effect of cyclophosphamide on tumor immunity. Strong growth inhibition of MH129 tumors by low-dose cyclophosphamide in the syngeneic immuno-competent but not immuno-incompetent nude mice, extensive intratumoral lymphocyte infiltration and disappearance of this anti-tumor effect by CD4⁺CD25⁺ T cell repletion collectively indicate that the anti-tumor effect of low-dose cyclophosphamide is mediated by its immuno-enhancing potential induced by CD4⁺CD25⁺ T cell depletion,

rather than its direct cytotoxic effect as an alkylating agent. In contrast, the similar anti-tumor effects of high-dose cyclophosphamide in mice and sparse intratumoral lymphocyte infiltration indicates that the anti-tumor effect of high-dose cyclophosphamide is solely comprised of the direct cytotoxic effect. Thus, we definitely demonstrate the difference in the effects of low- and high-doses of cyclophosphamide on anti-tumor immunity. Thus, low-dose cyclophosphamide enhances anti-tumor immunity by selectively depleting CD4⁺CD25⁺ regulatory T cells, while high-dose cyclophosphamide does not because of elimination of both effector and regulatory T cells. Our data suggest that 50% decreases in conventional, effector CD4⁺ T and CD8⁺ T cell numbers by low-dose cyclophosphamide are unlikely to affect overall immune reaction, but the >90% decreases seem to be critical.

Although the timing of cyclophosphamide injection to obtain the optimal result is controversial in other literatures (17,18), administration of cyclophosphamide after tumor inoculation is more efficacious than prior injection in this study. These data are similar to those in the recent report obtained with the agonistic anti-glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) antibody (DTA-1) (19). In contrast, the anti-tumor effect of anti-CD25 antibody-mediated CD4⁺CD25⁺ T cell depletion is higher when antibody is given before tumor cell inoculation than after tumor cell injection in the present and previous studies (15,16). This is at least partly because CD25 is also expressed on activated T cells (14). Thus, anti-CD25 antibody can eliminate not only regulatory cells but also activated effector cells (both CD4⁺CD25⁺) when administered after tumor cell inoculation. On the contrary, low-dose cyclophosphamide does not seem to affect activated CD4⁺CD25⁺ effector T cells. Anti-GITR antibody stimulates the activated CD4⁺CD25⁺ effector T cells (20). In this regard, use of cyclophosphamide or agonistic anti-GITR antibody appears to be more practical in a clinical setting.

Since recent studies show an increase in CD4⁺CD25⁺ T cells in patients with various cancers (21-23), suppression of number and/or function of CD4⁺CD25⁺ T cells may be critical for successful anti-cancer immunotherapy. It may be worthy noting here that selective suppression of CD4⁺CD25⁺ T cells has also been recently described in methotrexate (8) and fludarabine (24).

It should be emphasized here that the low-dose cyclophosphamide we used in this study (20 mg/kg) approximately corresponds to a dose of cyclophosphamide commonly used in cancer chemotherapy in humans (1000 mg/m²) (25). Furthermore, 200-300 mg/m² cyclophosphamide has widely been used as an immuno-potentiating agent in cancer immunotherapy (2). Therefore, it may be particularly important to scrutinize the effect of lower-dose cyclophosphamide (20 mg/kg or less) on immune function including number and function of CD4⁺CD25⁺ regulatory T cells in humans.

In conclusion, we here report that low-dose (20 mg/kg) cyclophosphamide selectively depletes CD4⁺CD25⁺ T cells, thereby enhancing anti-tumor immunity. These findings appear to be highly critical in terms of combined chemotherapy and immunotherapy for cancer treatment in humans. Further studies on elucidating the molecular mechanisms for cyclophosphamide-mediated suppression of regulatory T cells

will help us better understand regulatory T cell physiology and develop novel strategies for cancer treatment.

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