Mechanisms involved in synergistic anticancer effects of anti-4-1BB and cyclophosphamide therapy

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Abstract
Chemotherapy can precondition for immunotherapy by creating an environment for homeostatic lymphoproliferation and eliminating some of the suppressive immune networks. We found that combination therapy with anti-4-1BB and cyclophosphamide (CTX) produced synergistic anticancer effects in the poorly immunogenic B16 melanoma model in mice. The antitumor effect of the combination therapy depended mainly on CD8+ T cells, the 4-1BB–dependent expansion and differentiation of which into IFN-γ-producing CD11c+CD8+ T cells was enhanced by CTX. Anti-4-1BB induced a rapid repopulation of T and B cells from CTX-mediated lymphopenia. Anti-4-1BB protected naive T cells from repopulation of T and B cells from CTX-mediated lymphopenia. CTX treatment enhanced 4-1BB expression on CD4 and CD8 T cells, and CTX alone or in combination with anti-4-1BB effectively suppressed peripheral regulatory T cells. Our results indicate that anti-4-1BB and CTX can be practical partners in cancer therapy because CTX creates an environment in which anti-4-1BB actively promotes the differentiation and expansion of tumor-specific CTLs. [Mol Cancer Ther 2009;8(2):469–78]

Introduction
Tumors use various mechanisms to suppress antitumor immune responses. These include CD4+CD25+ regulatory T (Treg) cells, dendritic cells expressing indoleamine 2,3-dioxygenase, CD4+ T cells secreting Th2-type cytokines, soluble factors such as interleukin-10 and transforming growth factor β, and inhibitory cell surface molecules such as PD-1 and CTLA-4 (1–8). Successful immunotherapy against tumors, therefore, requires both the ability to overcome such immune network suppression and the marshaling of active tumor killing mechanisms simultaneously.

Tumor-specific T-cell responses can be enhanced by inducing lymphopenia using irradiation or treatment with anticancer chemotherapy (9–11). Lymphodepletion has been known to promote homeostatic T-cell proliferation by creating the space and cytokine milieu for T-cell expansion and also by increasing tumor antigen presentation (12–14). In addition, lymphodepletion seems to eliminate tumor-mediated tolerance by removing the immune cells that negatively regulate the antitumor response, although the beneficial immune cells may be depleted as well.

The DNA alkylating agent cyclophosphamide (CTX) is a chemotherapeutic agent used to treat various types of cancer and is well known to potentiate the immune response by removing Treg cells and blocking their function (15–18). 4-1BB is a T-cell surface receptor induced in an antigen-specific manner, and 4-1BB–mediated signaling by anti-4-1BB monoclonal antibody (mAb) results in enhanced antigen-specific immune responses (19–21). The efficacy of agonistic anti-4-1BB mAb has been well documented in tumor therapy, and its antitumor effect is mainly exerted by inducing expansion and differentiation of polyclonal tumor-specific CD8+ T cells (22, 23). 4-1BB was induced on human T cells on stimulation with DNA-damaging reagents such as γ-irradiation and chemotherapeutic agents, and cross-linking of 4-1BB with anti-4-1BB prolonged the survival of the cells (24).

We therefore hypothesized that anti-4-1BB and CTX may act as therapeutically synergistic partners against cancer: CTX creates an environment that facilitates the anti-4-1BB–mediated antitumor activities whereas anti-4-1BB provides...
the active killing mechanisms against the tumor. To test this hypothesis, we investigated the combined action of agonistic anti-4-1BB with CTX in the poorly immunogenic B16-F10 melanoma mouse model.

**Materials and Methods**

**Mice and Cell Lines**

C57BL/6 mice (6–8 wk old) were purchased from Charles River. Recombinase-activating gene-2–deficient (Rag2−/−), OT-1×RAG-1−/− mice on C57BL/6 background were purchased from The Jackson Laboratory. All mice were housed in a specific pathogen-free facility at the Immunomodulation Research Center, University of Ulsan, Ulsan, Korea. All animal experiments were done in accordance with approved protocols and the guidelines of the Immunomodulation Research Center Institutional Animal Care and Use Committee. B16-F10 melanoma cells were maintained in DMEM containing 10% fetal bovine serum (Life Technologies, Inc.), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

**Antibodies, Microbeads, and Peptides**

Hybridoma cells producing antimouse 4-1BB mAb (3E1) have been described (25). The hybridoma cell lines anti-CD8 (2.43) and anti-NK1.1 (PK136) were purchased from American Type Culture Collection. The antibodies were purified from hybridoma cell culture supernatant in our laboratory using protein G-columns (Sigma). Purified rat IgG was obtained from Sigma-Aldrich and served as a control antibody. The following mAbs were purchased by BD Pharmingen: FITC–, phycoerythrin (PE)–, and PE-Cy5–anti-CD8α (53-6.7); FITC–, PE-Cy5–, and biotin–anti-CD8β (Ly-3.2, 53-5.8); FITC–, PE–, and PE-Cy5–anti-CD4 (H129.19); FITC– and PE-anti-CD11c (HL3); PE–anti-B220 (RA3-6B2); PE–anti-IFN-γ (XMG1.2); purified anti-CD16/CD32 (2.4G2); and biotin-labeled CD25 (7D4). FITC-anti-CD25 (PC61), PE-anti-CD44 (IM7), FITC-anti-CD62L (MEL-14), and FITC-anti-CD69 (H1.2F3) were purchased from eBioscience. Streptavidin–, CD4–, and CD8–microbeads were obtained from Miltenyi Biotec. The tumor peptides gp100 (ITDQFVPSV), mMAGE (HNTQYCNL), mTyrosinase (FMĐGTMŚQV), and mTRP2 (VYĐFFVWL) were synthesized from Peptron.

**Tumor Challenge and Treatment with Anti-4-1BB mAb and CTX**

C57BL/6 mice were injected s.c. with 4 × 10⁵ B16-F10 melanoma cells under the skin on the dorsal surface on post-tumor injection (PI) day 0. Standard treatment involved i.p. administration of a single injection of 150 mg/kg CTX on PI day 0 and five injections of 100 μg of rat IgG or anti-4-1BB mAb (3E1), 5 d apart beginning on PI day 0, or some combination thereof. To examine the antitumor effects of combination therapy on established tumors, the standard therapy was given beginning either 5 or 10 d after tumor injection. Experiments were terminated when tumor size reached 20 mm in diameter, according to the guidelines of Institutional Animal Care and Use Committee of the Immunomodulation Research Center.

**Flow Cytometry and Cellular Kinetics**

Single-cell suspensions were prepared from tumor-draining lymph nodes (TDLN; inguinal lymph nodes). Cells were incubated with Fc blocker 2.4G2 for 5 min at 4°C and stained with specific antibodies. To discriminate among the naïve, effector, and memory CD4⁺ and CD8⁺ T cells, TDLN cells were triple-stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD62L mAb along with PE-Cy5–conjugated anti-CD4 or anti-CD8 mAb. To determine the cellular kinetics, viable TDLN cells were recovered, counted by hemocytometer, and stained with fluorescent-conjugated anti-CD4, anti-CD8, or anti-B220 mAb on the indicated days. The absolute number of each cell subset was calculated by multiplying the percentage of a given cell type, measured by flow cytometry, by the total number of viable cells (absolute number = percentage × total cells recovered). All samples were analyzed on a FACScan (BD Biosciences).

**Intracellular Staining of IFN-γ and Foxp3**

TDLN cells were stimulated with 50 μg/mL phorbol 12-myristate 13-acetate plus 500 ng/mL ionomycin for 1 h and cultured with 1 μmol/L brefeldin A (BD Pharmingen) for another 5 h. The stimulated cells were stained with FITC-conjugated anti-CD8 or anti-CD4 following Fc block with 2.4G2, fixed, and permeabilized with a Cytofix/cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions, and then incubated with PE-conjugated anti-IFN-γ. To detect Foxp3, TDLN cells were first surface-stained with PE-Cy5–anti-CD4 and FITC-anti-CD25 mAbs and then intracellularly stained with PE-anti-Foxp3 mAb according to the manufacturer’s instructions (eBioscience).

**CD107 Mobilization Assay**

EL4 cells were used as target cells by pulsing with 50 μg/mL of the tumor-associated peptides gp100, TRP2, tyrosinase, and MAGE-A1. Effector cells were prepared from the TDLNs of tumor-challenged mice. Viable cells were isolated, washed, and suspended at the appropriate concentration (2 × 10⁶/mL). The effector/target ratio was 1:2. To each well, the following were added in order: 1 μL of 2 mmol/L monensin (Sigma-Aldrich) in 100% ethanol, 100 μL of target cells, 10 μL of effector cells, and 1 μL of antibody to CD107α (BD Biosciences). The plates were centrifuged at 300 × g for 1 min to pellet the cells and then placed in an incubator at 37°C for 5 h. After incubation, the plates were centrifuged at 500 × g to pellet the cells and the supernatants were removed. Cell-cell conjugates were disrupted by washing the cells with PBS supplemented with 0.02% azide and 0.5 mmol/L EDTA. The samples were then mixed vigorously and stained with PE-anti-CD11c and PE-Cy5–anti-CD8.

**Depletion of CD8⁺ T and NK Cells In vivo**

The mice were injected i.p. with 400 μg of anti-CD8 (2.43) or anti-NK1.1 (PK136) antibody every 5 d for a total of five injections beginning on PI day 0. Rat IgG was given in the same way, as a control. The efficacy of immune subset depletion (>99%) with the use of these antibodies was confirmed by flow cytometry.
Cell Purification

Cell suspensions were prepared from the spleens and lymph nodes of Thy1.1 C57BL/6 mice and preincubated with Fc blocker 2.4G2 for 10 min at 4°C. The CD8+ or CD4+CD25+ T cells were further isolated by incubating the cells with CD8-microbeads or CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s directions. The purity of the CD8+ and CD25+ T cells was >95% by flow cytometry.

Preparation of Memory OT-1+ T Cells

For in vitro activation of OT-1 cells, CD8+ T cells were purified from OT-1 transgenic mice, stimulated with 1 μg/mL OVA257-264 peptides in RPMI medium for 24 h, and cultured with 20 IU/mL of recombinant human interleukin-2 for an additional 4 d. C57BL/6 mice received the in vitro activated OT-1 cells at a density of 1 × 10^7 per mouse. The mice were held for more than 35 d to generate memory OT-1 cells.

Results

Anti-4-1BB and CTX Synergistically Enhance the Antitumor Response

We tested the anticancer effect of anti-4-1BB and CTX, alone and in combination, in the s.c. B16 melanoma mouse model. Treatment with anti-4-1BB and/or CTX was begun on PI day 0, 5, or 10. CTX was given once and anti-4-1BB was given five times at 5-day intervals. Anti-4-1BB alone resulted in improved survival times but did not suppress the growth rate of the tumor. CTX alone transiently suppressed tumor growth until PI day 25 and improved the overall survival rate. The effects were statistically significant (P = 0.021 for PI day 42 and P = 0.037 for PI day 45 of tumor growth) only when the mice were treated from PI day 0 (Fig. 1A); both effects were completely lost when treatment was delayed until PI day 5 or 10 (Fig. 1A). Overall, treatment with anti-4-1BB or CTX alone was not sufficient to induce a strong antitumor response in the B16-F10 melanoma model.

By contrast, combination therapy with anti-4-1BB and CTX showed a synergistic anticancer effect (Fig. 1B, left). When treatment began on PI day 0, tumor size remained <5 mm (range, 0–10 mm) in diameter in more than 70% of tumor-bearing mice until PI day 50 (Supplementary Fig. S1).

Figure 1. Anti-4-1BB and CTX synergistically enhance the antitumor response. C57BL/6 mice were s.c. challenged with 4 × 10^5 B16-F10 melanoma under the skin of the dorsal surface. Treatments consisted of a single i.p. injection of CTX (150 mg/kg) on PI day 0, 5, or 10; 100 μg of anti-4-1BB mAb (3E1) every 5 d beginning on PI day 0, 5, or 10; or a combination of CTX and 4-1BB. Rat IgG (100 μg), given i.p. on the same schedule as 4-1BB, was used as the control. The mice were monitored daily to determine tumor volume and survival. A, tumor size (left) and survival rate (right) after CTX treatment. B, tumor size (left) and survival rate (right) after combination treatment (CTX + anti-4-1BB). C, percentage of tumor-free mice on PI day 100. Each group contained six mice and results are representative of three independent experiments. Points, mean; bars, SD.
The antitumor effect was reduced when treatment was begun on PI day 5 or 10 (Fig. 1B, left). Combination therapy also markedly prolonged survival times when treatment was begun on PI day 0: more than 80% of the mice survived until PI day 50. However, delayed treatment resulted in decreased survival rates (Fig. 1B, right).

The most notable result was that the combination therapy resulted in 20% to 30% tumor-free mice in this B16-F10 melanoma model following treatment begun on PI day 0 and 5% to 10% tumor-free mice following treatment begun on PI day 5 (Fig. 1C). There were no tumor-free mice among those that received the combined therapy beginning on PI day 10, although tumor size was reduced and survival rate improved (Fig. 1B and C). Further analysis provided more information about mice that received combination therapy. We divided mice with combination therapy into four groups according to tumor size on PI day 50. When combination treatment began on PI day 0, 25% of mice had complete remission, 35% had stable disease (<10 mm in diameter), 30% had partial response (>10 mm in diameter), and 10% had no response on PI day 50 (Supplementary Table S1).4 However, delayed treatment from PI day 5 or 10 decreased not only survival rates but also the proportion of mice that showed complete remission and stable disease on PI day 50, compared with mice that received combination therapy from PI day 0.

Taken together, these results show that the combination of anti-4-1BB and CTX synergistically induces an effective antitumor response that is strong enough to generate tumor-free mice in the B16-F10 melanoma model.

**CD8^+ T Cells Mediate the Antitumor Effects of Combined Therapy**

In 4-1BB-mediated cancer therapy, the antitumor efficacy of anti-4-1BB is mainly mediated by NK cells and CD8^+ T cells, and the effect is dependent on IFN-γ (23, 26–29). Therefore, we determined the role of NK and CD8^+ T cells in combination therapy by depleting CD8^+ T or NK cells. Depletion of NK cells partially reversed and depletion of CD8^+ T cells completely abolished the therapeutic effects of the combination therapy (Fig. 2A). The depletion of CD4^+ cells further enhanced the therapeutic effects of combination therapy (Supplementary Fig. S2).4 This result is consistent with our previous report (22).

In support of these results, we then measured IFN-γ production from CD4^+ and CD8^+ T cells from TDLNs (inguinal lymph node). On PI day 22, IFN-γ was produced in T cells at high levels following combination treatment begun on PI day 0. IFN-γ was enhanced in both CD4^+ and CD8^+ T cells by anti-4-1BB treatment alone (1.61 ± 0.33% and 3.65 ± 0.59%, respectively), compared with rat IgG–treated mice (0.30 ± 0.11% and 0.35 ± 0.13%, respectively), and also moderately increased by CTX treatment alone.
We next examined the lymphocytes that infiltrated into the tumor tissues on PI day 22. Anti-4-1BB treatment resulted in an initial decrease in lymphoid cell number of 25-fold that seen with CTX alone on day 22. Therefore, we examined the effect of anti-4-1BB on CTX-mediated lymphopenia in vivo. We determined the absolute numbers of CD4⁺ and CD8⁺ T cells and B220⁺ B cells in TDLN following treatment with anti-4-1BB and/or CTX and evaluated the kinetics of the T- and B-cell numbers. Consistent with a previous report (34), CTX treatment decreased the number of lymphoid cells to 1% of normal by day 4; cell numbers gradually increased thereafter, reaching normal levels by day 16. In the control IgG-treated group, lymphoid cell numbers in TDLN started to increase by day 12, peaked by day 16, and remained at that level. Treatment with anti-4-1BB alone produced a gradual increase in the number of lymphoid cells in TDLNs, peaking by day 24 at levels ~2-fold those seen with IgG (Fig. 3A). Combination anti-4-1BB and CTX treatment resulted in an initial decrease in lymphoid cell numbers similar to that seen with CTX alone; however, the number of cells was 25-fold that seen with CTX alone on day 4 (2.5×10⁶ versus 0.1×10⁶; Supplementary Table S2). The recovery of cell numbers was rapid, surpassing that of the rat IgG–treated mice around PI day 16, and finally becoming comparable with that of anti-4-1BB–treated mice by PI day 24 (Fig. 3A). The changes in B220⁺ B, CD4⁺ T, and CD8⁺ T-cell numbers followed a pattern similar to that of the total lymphocytes (Fig. 3B-D).

Figure 3. Anti-4-1BB induces rapid recovery from CTX-mediated lymphopenia. C57BL/6 mice were injected s.c. with 4×10⁶ B16-F10 melanoma cells under the skin of the dorsal surface. Treatment consisted of a single i.p. injection of CTX (150 mg/kg) on PI day 0 (gray arrow) and/or 100 μg of anti-4-1BB (3E1) i.p. every 5 d for a total of five doses (black arrow). Combination therapy involved a single CTX injection followed by the anti-4-1BB regimen. Rat IgG (100 μg), given on the same schedule as anti-4-1BB, was used as the control. Single-cell suspensions were prepared from TDLNs (inguinal lymph nodes) of each group of mice on the indicated days. TDLN cells were counted and stained with FITC-conjugated anti-CD4, anti-CD8, or anti-B220 mAb. Absolute numbers of each population were calculated by multiplying percentages measured by flow cytometry by total numbers of viable cells. The number of total lymphoid cells (A), B220⁺ (B), CD4⁺ T (C), and CD8⁺ T cells (D) in TDLN. Each group contained five mice. Points, mean; bars, SD.

Anti-4-1BB Induces Rapid Recovery from CTX-Mediated Lymphopenia

CTX induces lymphopenia with depletion of Treg cells (32), and 4-1BB signaling produces antiapoptotic effects in CD4⁺ and CD8⁺ T cells (33, 34). Therefore, we examined the effect of anti-4-1BB on CTX-mediated lymphopenia in vivo. We determined the absolute numbers of CD4⁺ and CD8⁺ T cells and B220⁺ B cells in TDLN following treatment with anti-4-1BB and/or CTX and evaluated the kinetics of the T- and B-cell numbers. Consistent with a previous report (34), CTX treatment decreased the number of lymphoid cells to 1% of normal by day 4; cell numbers gradually increased thereafter, reaching normal levels by day 16. In the control IgG-treated group, lymphoid cell numbers in TDLN started to increase by day 12, peaked by day 16, and remained at that level. Treatment with anti-4-1BB alone produced a gradual increase in the number of lymphoid cells in TDLNs, peaking by day 24 at levels ~2-fold those seen with IgG (Fig. 3A). Combination anti-4-1BB and CTX treatment resulted in an initial decrease in lymphoid cell numbers similar to that seen with CTX alone; however, the number of cells was 25-fold that seen with CTX alone on day 4 (2.5×10⁶ versus 0.1×10⁶; Supplementary Table S2). The recovery of cell numbers was rapid, surpassing that of the rat IgG–treated mice around PI day 16, and finally becoming comparable with that of anti-4-1BB–treated mice by PI day 24 (Fig. 3A). The changes in B220⁺ B, CD4⁺ T, and CD8⁺ T-cell numbers followed a pattern similar to that of the total lymphocytes (Fig. 3B-D).

(1.59 ± 0.31% and 1.53 ± 0.22%, respectively; Fig. 2B). By combining anti-4-1BB with CTX, IFN-γ production was markedly enhanced in T cells, more so in CD8⁺ T cells (8.2 ± 1.87%) than in CD4⁺ T cells (2.21 ± 0.46%; Fig. 2B). We also found that IFN-γ–producing CD8⁺ T cells were restricted to the CD11c⁺CD8⁺ T cells that were induced in an antigen-specific and 4-1BB–dependent manner, as we previously reported (30, 31). Triple staining of TDLN cells with anti-CD8, anti-CD11c, and anti-IFN-γ showed that 84.8 ± 5.5% of the IFN-γ⁺CD8⁺ T cells expressed CD11c molecules (Fig. 2C). We also found that CD11c⁺CD8⁺ T cells (22), known to be effectors of anti-4-1BB–mediated tumor suppression and markedly expanded by combination therapy, similarly appeared in spleen, peripheral blood, and bone marrow together with draining lymph nodes (Supplementary Fig. S3).

We next examined the lymphocytes that infiltrated into the tumor tissues on PI day 22. Anti-4-1BB treatment increased the infiltration of CD8⁺ T cells, especially effector CD8⁺ T cells (CD11c⁺CD8⁺ T cells and CD62LlowCD8⁺ T cells), compared with that of the control IgG-treated group (Supplementary Fig. S4). CTX treatment alone caused the infiltration of a few CD8⁺ T cells into the tumor tissue, although its treatment transiently suppressed tumor growth until PI day 25 and improved the overall survival rate. By contrast, the combination of anti-4-1BB with CTX synergistically increased the percentage of CD8⁺ T cells among tumor-infiltrating lymphocytes, and most of the CD8⁺ tumor-infiltrating lymphocytes were CD11c⁺CD8⁺ T cells with CD62Llow, which were the effectors of anti-4-1BB–mediated tumor suppression and markedly expanded by the combination therapy. These results indicate that the combination with anti-4-1BB and CTX facilitates the expansion and infiltration of effector CD8⁺ T cells, which produce the antitumor effects. To determine whether combination therapy can produce memory CD8⁺ T cells against the melanoma, we purified CD8⁺ T cells from the tumor-free mice on PI day 50 and adoptively transferred the indicated number of cells into Rag2⁻/⁻ mice that were injected s.c. with 4×10⁶ B16-F10 melanoma cells. The adoptive transfer of >5×10⁶ CD8⁺ T cells significantly suppressed tumor growth (P = 0.042 for PI day 20 and P = 0.028 for PI day 24; Fig. 2D). When we rechallenged the mice that are tumor-free for >50 days among the combination treatment group with B16F10 or unrelated tumor MC38, the growth of B16F10 was delayed, whereas MC38 grew aggressively, which was comparable between naïve and tumor-free mice (Supplementary Fig. S5). These results indicate that the combination of anti-4-1BB and CTX induces an antigen-specific memory against B16F10 melanoma. These results indicate that CD8⁺ T cells are the dominant mediator of the antitumor response of the combination therapy, and provide memory cells against the melanoma.
Taken together, these results indicate that treatment with anti-4-1BB promotes the repopulation of lymphoid cells in TDLNs following the induction of lymphopenia by CTX.

Combination Therapy Promotes the Expansion of Tumor Antigen–Specific CD8+ T Cells
Combination therapy induced a strong antitumor response in a CD8+ T-cell–dependent manner and promoted the repopulation of lymphocytes in TDLNs. Because 4-1BB is induced on T cells by antigen engagement, and CTX enhances the expression of 4-1BB (24, 35), we hypothesized that the repopulated T cells after CTX-mediated lymphopenia are preferentially tumor antigen–specific T cells. To test this hypothesis, tumor-challenged mice were treated with CTX and/or anti-4-1BB, and tumor antigen–specific CTLs were enumerated by assessing the number of CD8+ T cells positive for lysosomal-associated membrane protein-1 (lamp-1; CD107a) during coculture with tumor-associated antigen (TAA)–pulsed target cells (36). Lymphocytes were prepared from the TDLNs of the four different groups of mice on the days indicated; cocultured with EL4 cells pulsed with gp100, TRP2, tyrosinase, or MAGE-A1 peptide; and lamp-1 expression on the surface of CD8+ T cells was detected. Representative results on PI day 22 showed that the combination therapy effectively increased lamp-1+CD8+ T cells against each TAA (Fig. 4A).

The kinetics of the lamp-1+CD8+ T cells against each TAA in the TDLNs showed that CTX alone was no more effective in inducing the TAA-specific CD8+ T cells than rat IgG (Fig. 4B). Because CTX treatment improved the survival rate of tumor-bearing mice (Fig. 1A), this result suggested that antitumor responses mediated by CTX were primarily based on the direct suppression of the tumor. Anti-4-1BB alone markedly increased the lamp-1+CD8+ T cells against each TAA initially, reaching a maximum around PI day 16 and remaining at that level until PI day 28. However, combination treatment with anti-4-1BB and a single injection of CTX resulted in continued increases in lamp-1+CD8+ T cells against each TAA until PI day 28, with levels exceeding those produced by anti-4-1BB alone by around PI day 20 (Fig. 4B).

Total CD8+ T-cell numbers were comparable for the anti-4-1BB alone and combination therapy groups (1.2-fold). However, the levels of lamp-1+CD8+ T cells in the combination therapy group were 2.2-fold higher than those on the surface of CD8+ T cells was detected. Representative results on PI day 22 showed that the combination therapy effectively increased lamp-1+CD8+ T cells against each TAA (Fig. 4A).

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Collectively, these results show that although 4-1BB triggering alone is able to expand TAA-specific CD8+ T cells in vivo, the expansion rate of CD8+ T cells seems to be limited, most likely due to the cytokine sinks and space in TDLNs (37). In combination with CTX, however, anti-4-1BB is able to induce the continuous proliferation of TAA-specific CD8+ T cells.

of the anti-4-1BB alone group (Fig. 4C) and 60-fold higher than those of the CTX alone group (Fig. 4B and C). Moreover, most of the lamp-1+CD8+ T cells expressed CD11c molecules on their surface (Fig. 4D), and the expanded CD8+ T cells were cytotoxic against TAA-pulsed EL4 cells in the 4-h 51Cr release assay (Supplementary Fig. S6).

Anti-4-1BB in Combination Therapy Protects Naive T Cells from CTX and Induces Proliferation of Both Effector/Memory and Memory T Cells

We determined the subsets of T cells that were repopulated with combination therapy. We first examined the expression of 4-1BB on T cells following CTX treatment. As shown in Fig. 5A, CTX treatment indeed enhanced 4-1BB on the surface of CD4+ and CD8+ T cells. We next determined the absolute numbers of naive, effector, and memory types of CD4+ and CD8+ T cells as distinguished by the expression of CD44 and CD62L: CD44low/CD62Lhigh as naive T cells, CD44high/CD62Llow as effector/memory T cells, and CD44high/CD62Lhigh as central memory T cells.
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Figure 6. 4-1BB triggering does not protect the Foxp3+ Treg cells from CTX-mediated depletion. A and B, C57BL/6 mice were challenged with 4 × 10^5 B16-F10 melanoma by s.c. injection on the dorsal surface. The mice were injected i.p. with 100 μg of rat IgG or anti-4-1BB mAb (3E1) every 5 d for five doses and also received a single i.p. injection of CTX (150 mg/kg) on PI day 0. Single-cell suspensions were prepared from TDLNs of each group of mice on PI day 5 (A) and day 15 (B). TDLN cells were surface-stained with FITC-anti-CD25 and PE-Cy5-anti-CD4, and then intracellularly stained with PE-anti-Foxp3. Absolute numbers of each population were calculated by multiplying percentages measured by flow cytometry by total numbers of viable cells (C and D). CD4+ CD25+ Treg cells were purified from lymph nodes and spleens of Thyl.1.C57BL/6 mice and transferred i.v. into 4-1BB-deficient C57BL/6 mice (Thyl.2;5 × 10^6 per mouse). Simultaneously, the mice were injected s.c. with 4 × 10^5 B16-F10 melanoma cells and treated with anti-4-1BB and/or CTX. TDLN cells were isolated from each group of mice on PI day 5 (C) and day 15 (D) and stained with FITC-anti-Thy1.1, PE-anti-Foxp3, and PE-Cy5-anti-CD4. Results are representative of two independent experiments. Points, mean; bars, SD.

TDLN cells were prepared from four different groups of mice on PI day 22 and analyzed for the three subsets of T cells. CTX treatment selectively reduced the naïve phenotype of CD4+ and CD8+ T cells compared with effector/memory and central memory types of cells. These results suggest that CTX induces the apoptosis of naïve T cells rather than the central memory T cells, and that anti-4-1BB effectively protects the naïve T cells and amplifies the surviving memory T cells, which might include the tumor antigen–specific T cells (Fig. 5B). Thus, 4-1BB triggering effectively expands the effector/memory and central memory phenotype T cells independent of CTX treatment (Fig. 5B).

The T cells increased by anti-4-1BB in the presence of CTX could have originated from both the periphery and bone marrow. To clarify whether anti-4-1BB protected the peripheral T cells from CTX, lymph node cells were prepared from naïve Thyl.1.C57BL/6 mice and adoptively transferred into tumor-challenged Thyl.2 congenic C57BL/6 mice, which were then treated with anti-4-1BB and/or CTX. We analyzed the Thyl.1-positive T cells in TDLNs on PI day 20 and found that a majority (>98%) of the surviving Thyl.1-positive T cells were of the effector/memory phenotype (CD4^high^CD8^low^ or high, data not shown). We found a slight increase in the numbers of CD4+ and CD8+ T cells with anti-4-1BB or CTX treatment (Fig. 5C).

However, combination therapy markedly increased the transferred Thyl.1+ T cells, particularly CD8+ T cells (Fig. 5C). These results indicate that anti-4-1BB and CTX synergistically expand the effector/memory CD4+ and CD8+ T cells in the periphery.

To further characterize whether the increases in CD8+ T cells after anti-4-1BB and CTX treatment were antigen specific and whether the activated or memory CD8+ T cells selectively survived after CTX treatment, we prepared C57BL/6 mice bearing naïve or memory OVA-specific CD8+ T cells as described in Materials and Methods. The mice were immunized with the emulsified OVA and treated with CTX and/or anti-4-1BB. Analysis of OVA-specific CD8+ T cells on PI day 20 indicated that CTX treatment resulted in a 1.33-fold increase in naïve OVA-specific CD8+ T cells and a 3.1-fold increase in memory CD8+ T cells compared with rat IgG treatment (Fig. 5D, left and right). Anti-4-1BB increased naïve CD8+ T cells by 2.6-fold and memory CD8+ T cells by 3.5-fold (Fig. 5D, left and right). Combination therapy increased naïve CD8+ T cells by 4.2-fold and memory CD8+ T cells by 8.1-fold (Fig. 5D, left and right). CTX treatment more efficiently enhanced the response of memory CD8+ T cells, compared with naïve CD8+ T cells, in the presence or absence of anti-4-1BB, which indicates that naïve CD8+ T cells are more sensitive to CTX-mediated toxicity than memory CD8+ T cells.

Taken together, these results show that effector/memory T cells in the periphery selectively survive CTX-mediated depletion, and anti-4-1BB protects naïve T cells and amplifies the surviving T cells. Moreover, due to the induction of lymphopenia, the combination therapy seems to induce the continuous proliferation of CD8+ T cells that are reactive with the tumors.

4-1BB Triggering Does Not Protect Foxp3+ Treg Cells from CTX-Mediated Depletion

CTX is known to selectively deplete and dampen the function of CD4+CD25+Foxp3+ Treg cells in vivo (15–17). Because of the protective effects of 4-1BB signaling on the CTX-mediated depletion of T cells (Fig. 5) and the constitutive expression of 4-1BB on Treg cells, we questioned whether anti-4-1BB would protect Treg cells from CTX-mediated depletion. To answer this question, we first determined the absolute numbers of Foxp3+ Treg cells in tumor-bearing mice on PI days 5 and 15 of anti-4-1BB and/or CTX therapy. Treg cells were increased 5-fold by anti-4-1BB alone, reduced by >95% following CTX treatment, and slightly increased after CTX-mediated depletion by treatment with anti-4-1BB on PI day 5 (Fig. 6A). By PI day 15, however, the numbers of Treg cells in mice that had undergone CTX-mediated depletion combined with anti-4-1BB treatment were comparable to those of control rat IgG–treated mice (Fig. 6B).

To discriminate the peripheral Treg cells from regenerating Treg cells, the purified CD4+CD25+ Treg cells from Thyl.1.C57BL/6 mice were adoptively transferred into congenic Thyl.2.C57BL/6 mice, which were subsequently injected with tumors as well as anti-4-1BB and/or CTX. The
transferred Treg cells gradually increased in number in TDLNs after anti-4-1BB treatment alone on PI days 5 and 15, but not when the treatment included CTX (Fig. 6C and D). Because the Thy1.2 Treg cells of recipient mice showed patterns similar to the result of Fig. 6A and B (data not shown), these results clearly show that 4-1BB triggering does not rescue the peripheral Treg cells from CTX-mediated depletion, although Treg cells newly generated from bone marrow are increased by 4-1BB triggering when the CTX concentration becomes low in vivo.

Discussion

CTX has unique properties with regard to the immune system, including the direct killing of tumor cells, the suppression of Treg cells, and the depletion of peripheral CD4+ and CD8+ T cells. In view of these properties, we tested the antitumor effects of anti-4-1BB plus CTX in the poorly immunogenic B16-F10 melanoma mouse model, which was not effectively treated by agonistic anti-4-1BB alone. We found evidence that the combined therapy synergistically elicited an antitumor response in this model, even generating tumor-free mice (Fig. 1B and C). The antitumor effect of the combination treatment was mainly mediated by CD8+ T cells and partially by NK cells (Fig. 2A). Detailed analysis revealed that the combination treatment synergistically augmented the CD8+ T response against tumor antigens by increasing the number of CTLs that expressed CD11c molecules on their surface (Figs. 2C and 4D).

CTX decreased the tumor burden and depleted mainly the naïve T-cell compartment, whereas 4-1BB triggering protected naïve T cells and induced the expansion of effector/memory and memory T cells. In addition, the lymphopenia induced by CTX seemed to provide an environment for the continuous expansion of tumor-reactive CTLs (Fig. 4B). This explains why only the combination therapy led to long-term protection from the tumor compared with anti-4-1BB or CTX treatment alone (Fig. 1). Additionally, the tumor-free mice showed no signs of autoimmune responses such as depigmentation or anti-DNA antibody (data not shown); this could be the result of the unique property of in vivo 4-1BB triggering—the preferential activation of CD8+ T cells (20, 30, 38).

Having found that 4-1BB triggering expanded the population of effector/memory T cells as CTX-mediated depletion of lymphocytes (Fig. 5A and B), we then looked at whether the memory T cells in the periphery were selectively spared from the effects of the CTX treatment and amplified by 4-1BB triggering. By adoptively transferring Thy1.1 T cells into congenic Thy1.2 mice and by treating the mice bearing OVA-specific naïve or memory CD8+ T cells with anti-4-1BB and/or CTX, we clearly showed that 4-1BB triggering protected and amplified the peripheral T cells that survived the CTX treatment, and the memory T cells were preferentially spared (Fig. 5C and D). However, anti-4-1BB treatment did not protect Treg cells from CTX-mediated depletion (Fig. 6). This may be because Treg cells are more sensitive to CTX-mediated depletion than conventional T cells (15, 16). Because CTX depletes most of peripheral Treg cells, there are no Treg cells that can be repopulated by anti-4-1BB in the periphery, although Treg cells newly generated from bone marrow are increased by 4-1BB triggering when the CTX concentration becomes low in vivo. Here, we used Foxp3-based assay for characterization of Treg cells in mice. In contrast to induction of Foxp3 expression in human, mouse Foxp3 was expressed on Treg cells, which are generated in the thymus and differentiated from peripheral CD4+CD25+ naïve T cells and are anergic and suppressive (39–41). These indicate that Foxp3 is the only product known to be exclusively expressed in Treg of mice and is a useful marker to characterize the mouse Treg cells.

The combination therapy was strong enough to generate tumor-free mice in the B16-F10 melanoma model. Successful immunotherapy requires subversion of immunosuppressive networks that are derived from both the tumor and immune systems (42). Adoptive tumor-infiltrating lymphocyte therapy was successful when the host immune system was properly conditioned by lymphodepletion (9). Lymphodepletion by CTX may subvert the immunosuppressive factors and provide lymphoid space and a cytokine milieu that facilitate 4-1BB-mediated expansion and differentiation of tumor-specific CD11c+CD8+ T cells.

Taken together, these findings provide evidence that the combination of anti-4-1BB and CTX constitutes a potent antitumor therapy and generates long-lasting antitumor memory.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Synergistic Anti-Cancer Effect of 4-1BB and Cyclophosphamide


Molecular Cancer Therapeutics

Mechanisms involved in synergistic anticancer effects of anti-4-1BB and cyclophosphamide therapy

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