Mechanisms Involved in Synergistic Anticancer Immunity of Anti-4-1BB and Anti-CD4 Therapy

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Abstract
Anti-4-1BB–mediated anticancer effects were potentiated by depletion of CD4+ cells in B16F10 melanoma-bearing C57BL/6 mice. Anti-4-1BB induced the expansion and differentiation of polyclonal tumor-specific CD8+ T cells into IFN-γ–producing CD11c+CD8+ T cells. The CD4+ cell depletion was responsible for facilitating immune cell infiltration into tumor tissues and removing some regulatory barriers such as T regulatory and IDO+ dendritic cells. Both mAbs contributed to creating differentiation of CD8+ T cells into effective tumor killers. CD4+ T-cell depletion facilitated the infiltration of immune cells into the tumors and removed regulatory barriers such as T regulatory (Treg) and IDO+ dendritic cells. Both mAbs contributed to creating a CD8+ T-susceptible tumor microenvironment by inducing class I molecules on the surface of the melanoma cells.

Introduction
Immunotherapy is an approach to treating intractable diseases such as tumors, autoimmune diseases, transplantation rejection, and chronic infections by enhancing, suppressing, or modifying the immune system. One of the goals of immunotherapy is to achieve selective immune enhancement or suppression without deleterious effects on the global immunity of the host. 4-1BB is induced on the T-cell surface in an antigen-specific manner and directs antigen-specific immune responses; this provides a means to achieve such selectivity when it is used in immunotherapy (1, 2).

The efficacy of agonistic anti-4-1BB in tumor therapy has been well documented. Its therapeutic effects are mediated by enhancing natural killer (NK) and CD8+ T-cell activation and IFN-γ production (3–6). In a mouse melanoma model, however, injection of anti-4-1BB only partially suppressed B16-F10 subcutaneous tumors, although it preferentially increased the CD8+ T-cell response and IFN-γ production (6). To overcome some of the barriers that may inhibit anti-4-1BB–mediated anticancer effects, we have tested the efficacy of various reagents in combination with anti-4-1BB in B16 melanoma-bearing mice. Among the reagents, depleting anti-CD4 was the most efficient at increasing the effect of anti-4-1BB–mediated tumor therapy.

We describe here the therapeutic effect of an agonistic anti-4-1BB in combination with a depleting anti-CD4 monoclonal antibody (mAb) in a B16-F10 melanoma model. We found that anti-4-1BB treatment resulted in the polyclonal expansion and differentiation of CD8+ T cells into effective tumor killers. CD4+ T-cell depletion facilitated the infiltration of immune cells into the tumors and removed regulatory barriers such as T regulatory (Treg) and IDO+ dendritic cells. Both mAbs contributed to creating a CD8+ T-susceptible tumor microenvironment by inducing class I molecules on the surface of the melanoma cells.

Materials and Methods
Mice. IFN-γ–deficient (GKO) and Rag2–deficient C57BL/6 mice (6–8 weeks old) were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions in the Immunomodulation Research Center. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Ulsan.

Antibodies and peptides. Anti-m4-1BB hybridoma cells (3E1) were a gift from Dr. Robert Mittler (Emory University, Atlanta, GA) and the anti-NKG2D mAb (C7) was described previously (7). The anti-mCD4 (GK1.5) and anti-NK1.1 (PK136) hybridomas were obtained from American Type Culture Collection. The anti-mouse indoleamine 2,3-dioxygenase (IDO) mAb (mIDO-1) was generated in our laboratories. The following mAbs were purchased from BD PharMingen: FITC– or phycoerythrin (PE)-C5-anti-CD8α (53-6.7), FITC– or PE-C5-anti-CD8α (Ly-32, S3-58), PE– or PE-C5-anti-CD4 (H129.19), FITC-anti-CD25, FITC– or PE-anti-CD11c (HL3), PE-anti-CD11b (M1/70), FITC– or PE-anti-CD3 (145-2C11), PE-anti-B220 (RA3-6B2), PE-anti-NK1.1 (PK3E6), PE-IFN-γ (XMGI2.1), purified anti-CD16/CD32 (2.4G2), biotin-anti-CD40 (3/23), and biotin-anti-B220 (RA3-682). FITC– or PE-anti-NKG2D (CS5), PE-C5-anti-TCRβ; biotin-anti-F4/80 (BM8), biotin-anti-KLRG1 (MAFA, 2F1), and FITC– or PE-streptavidin were purchased from eBioscience. FITC– or PE-anti-PDCA-1 (JF05-IC4.2.1) were obtained from Miltenyi Biotec. Tumor peptides were synthesized by Peptron: mgp100 (ITDQVPFSV), nMAGE-A1 (HNTQYCNL), mTyrosinase (FMDGTMMSQV), and mTRP2 (YDDFFWVL).

Treatments. Mice were challenged s.c. on the back with 4 × 10^5 B16-F10 melanoma cells. For preventative therapy, mice were injected i.p. with 100 µg anti-4-1BB and/or 400 µg anti-CD4 every 5 days. To deplete NK cells, the cells were surface-stained with PE-conjugated anti-CD4, anti-CD8, or anti-B220 alone, or with PE-conjugated anti-CD11c and PE-C5-conjugated anti-CD8, and then fixed, permeabilized, and intracellularly stained with FITC-conjugated anti-BrdUrd (BD Bioscience).
Flow cytometry. Cells were incubated with the Fc blocker 2.4G2 for 10 min at 4°C and stained with specific antibodies to surface markers. The expression of MHC class I or IFN-γ was determined by staining with PE-conjugated anti-H-2Kb, anti-H-2Dd, or anti-IDO, and then stained intracellularly with FITC-conjugated BrdUrd mAb (BD Bioscience; C and stained with PE-conjugated anti-H-2Kb, anti-H-2Dd, or anti-IDO, and FITC-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD11c, or anti-NK1.1. The cytokines in the supernatants were quantified using a cytometric bead array kit (BD Bioscience). Cytokines. Tumor or spleen tissues were weighed and homogenized in PBS-containing protease inhibitors, and the supernatants were collected by centrifugation. The cytokines in the supernatants were quantified using a cytometric bead array kit (BD Bioscience).

Confocal microscopy. Sections of tumor tissue (6 μm thickness) were stained with PE-conjugated anti-H-2Kb, anti-H-2Kd, or anti-IDO, and mounted with Prolong Anti-fade (Molecular Probes). All sections were viewed and photographed using a laser-scanning confocal fluorescence microscope system (Fluoview FV500).

IDO in dendritic cells. CD11c+ dendritic cells were purified from the tumor tissue (6 μm thickness) were stained with PE-conjugated anti-H-2Kb, anti-H-2Kd, or anti-IDO, and mounted with Prolong Anti-fade (Molecular Probes). All sections were viewed and photographed using a laser-scanning confocal fluorescence microscope system (Fluoview FV500).

Results

CD4+ cell depletion enhances the antitumor response to anti-4-1BB. To enhance the 4-1BB-mediated antitumor CTL response, we tested a variety of reagents such as blocking anti-CTLA-4, blocking PD-1, depleting anti-CD25, or depleting anti-CD4 mAbs in combination with anti-4-1BB. A synergistic antitumor effect was observed when the anti-4-1BB was used in combination with depleting anti-CD4 in the B16-F10 melanoma model. Anti-4-1BB or anti-CD4 treatment by themselves slightly suppressed tumor growth and somewhat prolonged survival time (Fig. 1A). On the other hand, coadministration of anti-4-1BB and anti-CD4 not only successfully inhibited tumor growth but also increased survival (Fig. 1A). In this case, the mice survived for >50 days after inoculation of the B16-F10 melanoma, and the suppression of tumor growth was sustained. Tumor-bearing mice were also treated with anti-4-1BB and/or anti-CD4 from day 6 after tumor inoculation when the tumors were 3 to 5 mm in diameter. Anti-4-1BB or anti-CD4 mAb alone resulted in slight suppression of tumor growth when the tumors were 3 to 5 mm in diameter.
growth and somewhat delayed the death of the tumor-bearing mice (Fig. 1B). Again, coadministration of anti-4-1BB and anti-CD4 had a profound suppressing effect on tumor growth and increased the survival of the tumor-bearing mice (Fig. 1B; Supplementary Fig. S1).

Because CD8+ T cells are primarily responsible for successful tumor therapy (9–11), we first assessed the *in vivo* proliferation of CD8+ T cells in anti-4-1BB– and/or anti-CD4–treated mice. Proliferating cells obtained from TDLNs (inguinal lymph nodes) were identified by labeling the mice with BrdUrd on PI day 12. There were only a few proliferating CD8+, CD4+ T, and B220+ B cells (<0.5%) in mice treated with control rat antibody. Anti-4-1BB treatment preferentially increased the proliferation of CD8+ T cells (1.9 ± 0.45%) over CD4+ T (1.0 ± 0.29%) or B cells (0.2 ± 0.1%). Anti-CD4 treatment also enhanced the proliferation of CD8+ T cells (2.6 ± 0.5%), but not B cells (<0.1%). However, coadministration of anti-4-1BB with anti-CD4 resulted in the presence of a large number of proliferating CD8+ T cells; their proportion (9.7 ± 2.3%) was 5.1-fold higher than after anti-4-1BB treatment, and 3.7-fold higher than after anti-CD4 treatment (Fig. 1C).

As we previously reported (2), anti-4-1BB treatment induces a subset of CD8+ T cells, namely CD11c+CD8+ T cells, in an antigen-specific and 4-1BB–dependent manner. Therefore, we tested whether the proliferating CD8+ T cells included CD11c+CD8+ T cells. After staining the same BrdUrd-labeled lymphocytes with anti-CD11c, and with anti-CD8+ to exclude CD8αε+ dendritic cells, CD11c-CD8+ and CD11c+CD8+ T cells were separately gated on a CD8 versus CD11c dot plot and the proportion of proliferating cells in each population was determined. Of the CD11c-CD8+ T cells, 33.2 ± 4.7% were proliferating cells compared with only 10.4 ± 2.9% of the CD11c+CD8+ T cells (Fig. 1D).

Taken together, these results indicate that the strong antitumor immune response evoked by coadministration of anti-4-1BB and anti-CD4 results from robust proliferation of CD11c+CD8+ T cells.

**CD4+ cell depletion enhances the anti-4-1BB–mediated expansion and differentiation of CD8+ T cells.** We next analyzed the effect of anti-CD4 on the anti-4-1BB–mediated CD8+ T-cell response. We prepared TDLN cells from the four groups of mice 15 days after tumor challenge and determined the percentages and absolute numbers of CD11c+CD8+ T cells and IFN-γ–producing CD8+ T cells. Anti-4-1BB treatment preferentially increased the number of CD8+ T cells and eventually reversed the ratio of CD4+ to CD8+ T cells (data not shown). Anti-4-1BB increased the absolute number of CD8+ T cells 2.5-fold in the TDLNs, and anti-CD4 increased it 4.5-fold, compared with control IgG (Fig. 2A, bottom left). The combination therapy produced the highest number of CD8+ T cells; a 7- to 9-fold increase over the control IgG-treated group. Anti-4-1BB was, however, more effective in inducing
CD11c<sup>+</sup>CD8<sup>+</sup> T cells than anti-CD4 (23.3 ± 2.6% versus 2.3 ± 0.49% of CD8<sup>+</sup> T cells). The combination of the two mAbs resulted in a synergistic increase in the double-positive cells (35.7 ± 4.3% of CD8<sup>+</sup> T cells; Fig. 2A, top middle). The absolute number of CD11c<sup>+</sup>CD8<sup>+</sup> T cells was increased ~50-fold by the combination therapy compared with the control IgG- or anti-CD4–treated mice, and ~10-fold compared with the anti-4-1BB–treated mice (Fig. 2A, bottom middle). IFN-γ production was particularly noticeable because the combination therapy increased IFN-γ-producing CD8<sup>+</sup> T cells >50-fold and 8.5-fold compared with the control IgG- and anti-CD4–treated mice, respectively (Fig. 2A, right). It also produced ~3-fold more IFN-γ–producing CD8<sup>+</sup> T cells than anti-4-1BB alone. In the anti-4-1BB–treated mice, CD4<sup>+</sup> T cells also induced IFN-γ production (data not shown). As shown in Fig. 2A (top right), almost 50% of the CD8<sup>+</sup> T cells produced IFN-γ in the combination therapy and ~28% in the anti-4-1BB treatment. Although anti-CD4 treatment markedly increased the number of CD8<sup>+</sup> T cells in the TDLNs, the ratio of IFN-γ–producing CD8<sup>+</sup> T cells was comparable with that in the control IgG-treated mice (Fig. 2B, top right), indicating that anti-CD4 is not involved in CD8<sup>+</sup> T cell differentiation. However, the anti-4-1BB treatment not only increased the number of CD8<sup>+</sup> T cells, but also promoted the differentiation of CD8<sup>+</sup> and CD4<sup>+</sup> T cells into IFN-γ–producing effector T cells (Supplementary Fig. S2).

Due to the enhanced CD8<sup>+</sup> T response by anti-4-1BB treatment, we determined the levels of cytokines in tumor tissues and in the spleen. In tumor tissues, anti-4-1BB or anti-CD4 increased the production of proinflammatory cytokines such as IFN-γ, tumor necrosis factor-α (TNF-α), IL-12p70, and MCP-1 with the highest increase in the combination therapy. IL-6 and IL-10 levels were decreased or not altered by the above treatments (Fig. 2B). In the spleen, the level of IFN-γ was significantly increased by the anti-4-1BB or combination therapy. TNF-α production was enhanced only by the anti-4-1BB treatment. No changes in the levels of other cytokines were statistically significant (Supplementary Fig. S3). These results suggest that anti-4-1BB and anti-CD4 treatments promote type 1 cellular immune responses in the tumor tissues.

To assess the involvement of anti-4-1BB and anti-CD4 in the differentiation of CD8<sup>+</sup> T cells into CD11c<sup>+</sup>CD8<sup>+</sup> T cells more stringently, purified CD8<sup>+</sup> T cells were adoptively transferred to syngeneic Rag2<sup>−/−</sup> and C57BL/6 mice; melanomas were induced and the mice were exposed to the four different regimens: control IgG, anti-4-1BB, anti-CD4, and anti-4-1BB plus anti-CD4. The formation of CD8<sup>+</sup> T cells was mainly dependent on anti-4-1BB, and the depletion of CD4<sup>+</sup> cells had almost no effect (Fig. 2C).

Taken together, our findings indicate that anti-4-1BB treatment was able to induce the proliferation and differentiation of CD8<sup>+</sup> T cells, as well as the production of cytokines, including IFN-γ, TNF-α, MCP-1, and IL-12p70. On the other hand, anti-CD4 treatment markedly increased the number of CD8<sup>+</sup> T cells, but did not promote the formation of IFN-γ–producing cells. Therefore, anti-CD4 treatment tended to induce the proliferation of CD8<sup>+</sup> T cells rather than their differentiation into effector cells. Anti-4-1BB treatment eventually generated more IFN-γ–producing CD11c<sup>+</sup>CD8<sup>+</sup> T cells than anti-CD4 (Fig. 2A). These results indicate that depletion of CD4<sup>+</sup> cells facilitates and potentiates the anti-4-1BB–mediated expansion and differentiation of CD8<sup>+</sup> T cells.
CD11c+CD8+ T cells are effectors of anti-4-1BB-mediated tumor suppression. We tested whether the combination therapy increased the number of tumor-associated antigen (TAA)-specific CTLs. We used four different TAAs: gp100, tyrosinase-related protein 2 (TRP2), tyrosinase, and melanoma-associated Ag A1 (MAGE-A1) to determine whether the 4-1BB-mediated expansion of CTLs is polyclonal and whether the expansion involves all tumor-specific CTLs, not just certain antigen-specific CTLs. Tumor antigen–specific CTLs were enumerated by assessing the number of CD107α (lysosomal-associated membrane protein-1)–positive CD8+ T cells during coculture with TAA peptide-pulsed target cells (8). Lymphocytes were prepared from the TDLNs of the four groups of mice on PI day 15 and cocultured with gp100, TRP2, tyrosinase, or MAGE-A1 peptide-pulsed EL4 cells at a 2:1 ratio in the presence of monensin and anti-CD107α mAb for 5 h. Flow cytometric analysis showed that CD107α+ positive cells were barely detectable on the surface of CD8+ T cells from the rat IgG-treated mice (0.3–0.4%) but were present against each of the four TAAs at a level of 0.7% to 1.8% in the anti-4-1BB– or anti-CD4–treated mice. However, in the combination therapy group, >5% of the total cells were CD107α+CD8+ T cells against each of four TAAs (Fig. 3A). Anti-4-1BB treatment seemed to be more effective than anti-CD4 for producing antigen-specific killers (Fig. 3B). In the combination therapy, the proportion of CD107α+ positive cells to each of four TAAs among the CD8+ T cells reached around 12% (Fig. 3B). Specific lysis assays yielded results in agreement with the observed CD107α+CD8+ T-cell frequencies (Supplementary Fig. S4).

The absolute numbers of CTLs in the TDLNs specific for each tumor antigen were determined (Fig. 3C). In the anti-4-1BB– or anti-CD4–treated mice, the frequency of antigen-specific CTLs was 3- to 10-fold higher than in the control IgG-treated mice, whereas the combination therapy increased the number of the CD107α+ cells >40-fold against all the TAAs tested. Reanalysis of the CD107α+ cells in the CD11c+CD8+ and CD11c+CD8− T subsets of the mice given the combination therapy again supported the hypothesis that CD11c+CD8+ T cells are the effectors of anti-4-1BB–based tumor therapy because most of the CD107α+CD8− T cells were restricted to the CD11c+CD8+ T cells (Fig. 3D).

We conclude therefore that the suppression of tumor growth by anti-4-1BB treatment is primarily the consequence of the extensive increase of tumor-specific CTLs that express CD11c molecules on their surface, and that the CTLs expanded by anti-4-1BB treatment are polyclonal. Depleting CD4+ cells again synergistically enhanced the anti-4-1BB–mediated expansion of CTLs.

Selective accumulation of CD11c+CD8+ T cells in tumor tissue. We examined the phenotype of the CD8+ T cells that infiltrated the tumor tissues on PI day 15. There were only a few CD8+ T cells among the TILs in the control IgG-treated mice. However, anti-4-1BB treatment not only increased the infiltration of CD8+ T cells, but most of the CD8+ TILs were CD11c+CD8+ T cells. Anti-CD4 treatment alone increased the infiltration of various types of immune cells, and, unexpectedly, 17.1% of the TILs were CD11c+CD8+ T cells, although this cell population was rarely detected in TDLNs. It seems that CD11c+CD8+ T cells are generated during normal immune responses and rapidly migrate to target tissues. By combining anti-4-1BB with anti-CD4 mAb, the infiltration of CD8+CD11c+ T cells was markedly increased, and they became to represent nearly 80% of all the TILs (Fig. 4A).

When we calculated the absolute numbers of each cell population in tumor tissue, each type of treatment yielded a distinct profile of cell infiltration. Anti-CD4 seemed to greatly facilitate the infiltration of a variety of immune cells, including CD3+ T and NK cells, monocytes/macrophages, and dendritic cells, whereas anti-4-1BB treatment preferentially increased the infiltration of CD8+ T cells, particularly CD8+CD11c+ T cells (Fig. 4B). The absolute number of tumor-infiltrating immune cells in the anti-CD4 group was 8- and 4-fold more than that in the control IgG- and anti-4-1BB–treated groups, respectively, indicating that CD4+ cells...
constitute a barrier to immune cell infiltration. Even the absolute number of CD11c+CD8+ T cells with anti-CD4 treatment was 2-fold more than with anti-4-1BB treatment. Again, the combination therapy greatly enhanced the infiltration of CD11c+CD8+ T cells into tumor tissues. These were rarely found in the rat IgG-treated control mice (Fig. 4B).

We compared the gene expression profile of the CD11c+CD8+ T cells with that of the CD11c−/CD8+ T cells in the rat IgG-treated control mice (Fig. 4B).

We tested for surface expression of NKG2D and KLRG1 by flow cytometry. TDLN cells from the anti-4-1BB and/or anti-CD4–treated mice received 400 μg anti-NK1.1 mAb on P1 day 10, and were also given 500 μg anti-NKG2D mAb from P1 day 0 or 10 every 5 d, rat IgG as a control. The mice were monitored every day to measure the growth of tumors. Points, mean (n = 5 mice per group in A and B and n = 10 mice per group in C and D); bars, SD. All results are representative of three independent experiments.

Figure 5. KLRG1+NKG2D+CD11c+CD8+ T cells become the tumor-infiltrating effector T cells of anti-4-1BB therapy. Tumor-challenged mice were treated with antibodies as described above. On P1 day 15, TDLN cells (A) and TILs (B) were stained with antibodies against KLRG1 or NKG2D as well as PE-Cy5-conjugated anti-CD11c and PE-conjugated anti-CD4 mAbs. All samples were subsequently analyzed on FACS. C, tumor-challenged mice were treated with anti-4-1BB and/or anti-CD4, rat IgG as a control. Some mice received anti-4-1BB plus anti-CD4 mAb were further treated with 400 μg anti-NK1.1 (PK136; dNK) or 500 μg anti-NKG2D (C7) mAb from P1 day 10 every 5 d. D, anti-4-1BB and anti-CD4–treated mice received 400 μg anti-NK1.1 mAb on P1 day 10, and were also given 500 μg anti-NKG2D mAb from P1 day 0 or 10 every 5 d, rat IgG as a control. The mice were monitored every day to measure the growth of tumors. Points, mean (n = 5 mice per group in A and B and n = 10 mice per group in C and D); bars, SD. All results are representative of three independent experiments.
Anti-4-1BB alone was less effective than depletion of CD4+ T cells. To confirm the role of NK2GD on the T cells, tumor-bearing mice were given anti-NK1.1 mAb on PI day 10 along with anti-NK2GD on PI day 0 or 10. In the absence of NK cells, blocking NK2GD decreased the antitumor effect by 21% to 26%, indicating that NK2GD is active in the CD11c+CD8+ T cell–mediated antitumor effects and contributes to 20% to 26% of the CTL activity in the tumors (Fig. 5D).

We have thus provided evidence that NK2GD+KLRG1+CD11c+CD8+ T cells preferentially infiltrate into tumor tissue and inhibit tumor growth. We conclude that the anti-4-1BB–mediated differentiation of CD8+ T cells yields NK2GD+KLRG1+CD11c+CD8+ T cells and that their infiltration is amplified by depleting CD4+ T cells.

Anti-4-1BB or/and anti-CD4 change the tumor microenvironment. We next investigated possible changes of the tumor microenvironment induced by the combination therapy. We first asked whether combination therapy enhanced the expression of MHC class I molecules on the melanoma cell surfaces because effective tumor killing by CD8+ T cells requires antigen presentation. The combination therapy proved very effective in inducing both H-2Kb and H-2Db molecules on the melanoma cells, Expression in the combination therapy reached 88.5% for H-2Kb and 98.1% for H-2Db, whereas the control IgG–treated group gave only 1.5% for H-2Kb and 10.1% for H-2Db. Remarkably, depletion of CD4+ T cells was also quite effective in inducing the expression of H-2Kb (46.1% for H-2Kb) and 64% for H-2 Db). Anti-4-1BB alone was less effective than depletion of CD4+ T cells in inducing them (4.5% for H-2Kb and 24.2% for H-2Db; Fig. 6A and C).

In view of the fact that almost all the melanoma cells express IFN-γR (Fig. 6A) and that class I molecule induction is almost at a basal level in IFN-γ KO (GKO) mice (Fig. 6A, bottom), the production of IFN-γ inside the tumors must play a major role in inducing the expression of H-2Kb and H-2Db.

Depletion of CD4+ cells may result in the elimination of CD4+CD25+ Treg- and IDO-producing dendritic cells, which are known to suppress antitumor immunity (14–16). As expected, anti-CD4 completely depleted both CD4+ effector and regulatory T cells in the TDLNs and tumor tissues (Supplementary Fig. S5A). Anti-CD4 depleted 50% to 70% of the plasmacytoid dendritic cells that expressed PDCA-1 (Supplementary Fig. S5B), and markedly reduced the number of IDO-expressing dendritic cells (Fig. 6C).

In agreement with these results, IDO+ cells were readily detected in TDLN sections of the rat IgG- or anti-4-1BB–treated mice, but were barely detectable in the mice treated with anti-CD4- or anti-4-1BB plus anti-CD4 (Fig. 6D).

Depletion of Treg by anti-CD25 showed only mild therapeutic effect, which seemed to be due to the additional depletion of activated T cells that expressed CD25. The combination of anti-4-1BB with 1-MT had a limited therapeutic effect on B16-F10 melanoma model (Supplementary Fig. S6A). This may be due to the fact that 1-MT inhibits the NK activity (17). Blockade of transforming growth factor β along with anti-4-1BB definitely suppressed the tumor growth, but showed a reduced survival rate (Supplementary Fig. S6B).

We conclude that anti-CD4 treatment strengthens the anti-4-1BB–mediated CD8+ T-cell response by inducing the formation of class I molecules on tumor cells and removing IDO+ dendritic cells and Treg cells.
Discussion

4-1BB is induced on the T-cell surface in an antigen-specific manner. The 4-1BB–mediated therapeutic effect, therefore, is an antigen-driven phenomenon. 4-1BB triggering could provide the selectivity of immunotherapy and minimize adverse effects on global host immune responses. 4-1BB alone, however, has a limited therapeutic effect against highly metastatic nonimmunogenic tumors such as the B16-F10 melanoma. This limitation seems to arise from the regulatory mechanisms of the host immune system itself. When we removed some of the regulatory barriers by anti-CD4, the antitumor effect was remarkably enhanced. We have shown that all aspects of the antitumor activities were synergistically enhanced by the combination therapy: proliferation of CD8+ T cells, CD8+ T-cell differentiation, production of proinflammatory cytokines, expansion of polyclonal cytotoxic effectors, infiltration of effector CD8+ T cells into tumor tissues, and induction of a susceptible tumor microenvironment. More importantly, these factors were translated into a synergistic antitumor effect.

The results of the present investigation deliver the strong message that the immune system contains multiple layers of CD4+ cell-mediated regulatory mechanisms against CD8+ T-cell–mediated antitumor immunity. CD4+ cells inhibited the expansion and differentiation of antitumor CD8+ T cells, and blocked the infiltration of immune cells into tumor tissues (Figs. 2–4). Interestingly, depletion of CD4+ cells was very effective in inducing the formation of MHCI class I molecules on the tumor cells in vivo (Fig. 6). This phenomenon may be related to the massive infiltration of various immune cells into the tumors and the production of IFN-γ in the tumor microenvironment.

Induction of high level of IFN-γ was shown to impair CD8+ T-cell memory due to the apoptosis of CD4+ T cells (18). We examined a potential role of anti-4-1BB in the generation of the memory CD8+ T cell by transferring OVA-specific CD8+ T cells into rag2−/− mice and found that anti-4-1BB could generate the memory CD8+ T cells in the absence of CD4+ T cells. Anti-4-1BB–mediated induction of IL-7 and IL-15 in CD8+ T cells seem to provide some of the helping functions of CD4+ T cells.5

It was remarkable that 4-1BB stimulation alone drove the differentiation and expansion of CD8+ T cells in an antigen-specific manner in the absence of CD4+ T cells (Figs. 2D and 3B). This is in line with results that suggested that 4-1BB triggering provides certain signals supposed to be provided by CD4+ helper T cells (19, 20). 4-1BB–dependent and antigen-driven expansion of CD11c+CD8+ T cells has been shown in herpes simplex virus-1 infection (21), autoimmune disease (2, 22), and B16 tumor models (current studies). CD11c+CD8+ T cells have dual regulatory roles: They suppress antigen-specific CD4+ T cells by an IDO-dependent mechanism in autoimmune disease models (2), and they themselves become effective CTLs in viral infection and tumor models. The origin and developmental requirements of such CD11c+CD8+ regulatory T cells remain to be determined.

All the mice received the combination therapy eventually died around PI days 70 to 80. Further delay of the combination therapy provides little survival advantage in spite of enhanced CD11c+CD8+ T response in TDLNs.

Anti-4-1BB is known to induce IDO in dendritic cells and macrophages (2, 22), and this generates a barrier to tumor therapy. Removing CD4+ cells eliminated ~70% of the IDO-producing cells and all the Treg cells, and this may contribute to the enhanced antitumor outcome. Yu et al. (14) showed that local depletion of CD4+ cells led to the eradication of established tumor and development of long-term antitumor immunity.

Anti-4-1BB therapy has shown to be effective in various tumor models (5, 19, 23). Anti-4-1BB has been also used in combination with IL-12–expressing dendritic cell (24), human telomerase reverse transcriptase gene therapy (25), dendritic cell–based vaccine (4), IL-12 gene therapy (3), and radiation (26). Soluble 4-1BB ligand in combination with IL-12 gene therapy produced potent antitumor response against hepatic colon carcinoma model (27). Induction of potent antitumor effect by the use of single-chain Fv of anti-4-1BB is another important approach (28, 29).

NK2GD and KLRG1-positive CD11c+CD8+ T cells preferentially accumulated inside tumor tissues. Significance of KLRG-1 expression (12) remains to be determined. NK2GD may function as a costimulatory receptor or it may provide the CD8+ T cells with MHC-independent killing capability (13, 30, 31), although this aspect requires further investigation of the roles of authentic ligands such as RAE-1, H60, and MULT-1 (13, 32, 33).

Current studies suggest that one single type of immunotherapy may not be able to deal with all facets of tumor progression, and tumor- and immune system–derived regulatory barriers. Therefore, a rational combination of synergistic immunotherapies could provide a successful clinical approach to human cancers. The recent treatment of melanoma by adoptive TIL transfer in nonmyeloablative lymphodepleting conditions may be another example of overcoming tumor escape mechanisms and regulatory barriers (34, 35). Considering the difficulties involved in preparing a large number of TILs in vitro in the adoptive therapy, new therapeutic modalities that achieve such an antitumor effect by injecting combinations of immunotherapeutics need to be developed.

Acknowledgments

Received 3/20/2007; revised 5/31/2007; accepted 7/2/2007.

Grant support: NIH grants RO1EY013325, KRF-2005-201-E00008, and KRF-2005-084-E00001, Innovative Research Award from Arthritis Foundation, Atlanta, GA, and Science Research Centre funds to the Immunomodulation Research Center, from Korea Science and Engineering Foundation.

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