Combination Therapy with Anti–CTL Antigen-4 and Anti-4-1BB Antibodies Enhances Cancer Immunity and Reduces Autoimmunity

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Abstract

The majority of cancer antigens identified thus far have limited expression in normal tissues. It has been suggested that autoimmune disease is a necessary price for cancer immunity. This notion is supported by a recent clinical trial involving an anti–CTL antigen-4 (CTLA-4) antibody that showed significant clinical responses but severe autoimmune diseases in melanoma patients. To selectively modulate cancer immunity and autoimmunity, we used anti-CTLA-4 and anti-4-1BB antibodies to treat mice with a preexisting cancer, MC38. The combination of the two antibodies led to CD8 T-cell-mediated rejection of large established MC38 tumors and long-lasting immunity to the same tumor cells, although the same regimen was not effective for B16 melanoma. More importantly, whereas individual antibodies induced inflammation and autoimmune manifestations, combination therapy increased cancer immunity while reducing autoimmunity. The reduction of autoimmune effects correlates with an increased function of regulatory T cells. Our results suggest a novel approach to simultaneously enhance cancer immunity and reduce autoimmunity. (Cancer Res 2006; 66(14): 7276-84)

Introduction

The majority of cancer antigens identified thus far are derived from genes with some expression in normal tissues (1–4). Thus, cancer immunity is intricately linked to autoimmunity. A central issue in cancer immunotherapy is how to enhance cancer rejection without triggering severe autoimmune diseases. It is possible that autoimmune diseases are intrinsically linked to cancer immunity (4–6). Alternatively, these two can be selectively modulated as the immunity that leads to the destruction of normal tissue may differ from that which causes cancer rejection (7–9). These distinct requirements were revealed using mice with targeted mutations of one or more arms of immune effector mechanisms (8, 9), suggesting that it is theoretically possible to selectively modulate cancer immunity over autoimmunity. However, a generally applicable approach has not been reported. Moreover, essentially all studies that address the distinct requirements for autoimmune destruction and tumor rejection have used a model of melanoma-related hypopigmentation. It is still unclear whether autoimmunity to internal vital organs follows the same rules.

Antibodies against costimulatory molecules, such as CTL antigen-4 (CTLA-4) and 4-1BB, have emerged as potentially effective therapeutics for immune protection against a host of tumors (10–16). Studies in animal models have indicated that the antitumor response elicited by anti-CTLA-4 monoclonal antibody (mAb) is, at least in part, due to an antigen-specific T-cell response against normal "self"-differentiation antigens (17, 18). The tendency of anti-CTLA-4 antibodies to exacerbate autoimmune diseases is well documented in the mouse (19–22). This notion was further corroborated and proven to be a major limitation in more recent human trials in which the patients developed severe autoimmune manifestations that required discontinuation of treatment (23). On the other hand, cancer therapeutic anti-4-1BB mAbs have been shown to abrogate the development of autoimmune diseases in lupus-prone mice (24, 25).

The fact that anti-4-1BB mAbs can both stimulate antitumor responses and decrease autoimmune manifestations raises the intriguing possibility that the combination of this antibody with anti-CTLA-4 mAb may result in cancer rejection without autoimmunity. In this study, anti-CTLA-4 and anti-4-1BB were combined to induce rejection of large established tumors. The evidence suggests that, although individual antibodies both cause autoimmune manifestations and/or inflammation to selective organs, a combination of the two antibodies drastically increased cancer immunity while reducing inflammation and autoimmune effects. Moreover, a combination of the two antibodies increased the regulatory function of $CD4^+CD25^+$ T cells. Our results challenge the notion that autoimmunity is a necessary price for cancer immunity.

Materials and Methods

Antibodies. Anti-4-1BB mAb-producing hybridoma 2A (26) has been kindly provided by Dr. Lieping Chen (The Johns Hopkins University, Baltimore, MD). Anti-CTLA-4 mAb-producing hybridoma 4F10 (27) was a gift from Dr. Jeff Bluestone (University of California, San Francisco, CA). Anti-human CTLA-4 antibody L3D10 has been described in our recent reports (28, 29). Both anti-4-1BB and anti-CTLA-4 mAbs were purified from supernatant by a protein G column. Hamster and rat IgG were purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Hybridomas that secrete depleting antibodies specific for NK1.1 (PK136), CD4 (GK1.5), and CD8 (2.4.3) were purchased from American Tissue Culture Collection (Manassas, VA). The anti-4-1BB antibody 2A was biotinylated according to an established procedure. Fluorochrome-conjugated anti-CTLA-4, CD4, and CD25 were purchased from BD PharMingen (La Jolla, CA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Experimental animals. Six- to 8-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Mice with a knock-in of the human CTA-4 locus and their use for testing therapeutic effects of anti-CTLA-4 antibodies have been described (28). Animals were maintained under pathogen-free conditions in accordance with federal guidelines.

Cell lines and tumorigenicity assay. C57BL/6 colon cancer MC38 cells were purchased from American Tissue Culture Collection. MC38 cells (5×10^5) suspended in serum-free RPMI (100 μL) were injected s.c. in the flanks of mice. Starting at either day 2 (minimal disease model) or day 14 (large established tumor model), the tumor-bearing mice received three weekly injections of either hamster (800 μg/mouse/injection) plus rat (200 μg/mouse/injection) IgG, anti-CTLA-4 mAb 4F10 (800 μg/mouse/injection), anti-4-1BB antibody 2A (200 μg/mouse/injection), or both antibodies. The doses of individual antibodies were based on preliminary studies that show optimal protection. Tumor size and incidence were determined every 2 to 5 days by physical examination.

Proliferation assay. $CD4^+CD25^+$ cells were purified from pooled spleens and lymph nodes of antibody-treated mice to use as suppressor cells. $CD4^+CD25^-$ cells from normal mice were used as responder cells. $CD4^+CD25^-$ responder cells were cultured at 5×10^4 cells per well with 1 μg/mL soluble anti-CD3 mAb and 1×10^5 irradiated spleen cells from wild-type mice. $CD4^+CD25^+$ cells were added at 2-fold titrations. Cells were cultured for 69 to 72 hours, and [^{3}H]TdR was added at 1 μCi/well for the last 6 hours of culture. Cells were harvested and counted on a β-counter.

Flow cytometry. Lymphocyte subsets were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson Co., Mountain View, CA). With exception of anti-CTLA-4 antibody, all antibodies were incubated with the spleen cells at 4°C for 30 to 60 minutes. The unbound antibodies were washed away with PBS containing 1% FCS and 0.01% sodium azide. To analyze the expression of CTLA-4 on the surface of regulatory T cells (Treg), the spleen cells were incubated with 1 μg/mL phycoerythrin-conjugated 4F10 or isotype control in the presence of 0.1 μg/mL anti-CD3 and 1,000-fold excess of hamster IgG to block nonspecific binding. After washing away the unbound antibodies, the cells were placed at 4°C for staining with biotinylated anti-4-1BB antibodies and APC-conjugated streptavidin. The Treg were marked by anti-CD4-FITC and anti-CD25-cychrome. Foxp3 staining was carried out using fluorochrome-conjugated anti-Foxp3 (eBiosciences, San Diego, CA) according to the manufacturer's published protocol.

Depletion of lymphocyte subsets *in vivo*. *In vivo* depletion was achieved by injection of anti-CD4 (0.5 mg/injection/mouse), anti-CD8 (0.5 mg/mouse/injection), and anti-NK1.1 (0.1 mg/injection/mouse) on days 9, 12, and 16 after tumor cell inoculation.

Detection of anti-dsDNA or anti-antibody antibodies. Anti-DNA antibodies were measured by ELISA according to the published procedure (24). Similar methods were used to detect antibodies against 4F10 and 2A.

Immunofluorescent staining of frozen renal sections for glomerular antibody and complement deposition. Frozen sections of kidney were prepared at given time points after tumor cell inoculation and fixed in acetone. After blocking with 10% normal goat serum, the sections were stained with rhodamine-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-mouse C3 antibodies (ICN Biomedicals, Inc., Aurora, OH). A pathologist examined all slides for the presence or absence of IgG and C3 in a blinded fashion.

Histology. Internal organs from tumor-bearing mice receiving different antibodies were fixed with 10% formalin. The fixed tissues were sectioned and stained by H&E. A pathologist examined all samples for tumor metastasis and inflammation in a blinded fashion. The pathology score was based on the size and number of inflammatory foci as detailed in the figure legends.

Statistical methods. Summary plots were first produced for the data from the tumorigenicity assays. To assess trends over time, linear random effects models were fitted to the data. The combined effects of anti-CTLA-4 and anti-4-1BB on tumor growth were tested via a three-way interaction with day. ANOVA was used to assess the effects of the combination therapy on the production of anti-DNA antibodies and lupus-like pathology in the kidney. The proportions of mice with liver metastasis for each of the treatment combinations were compared via logistic regression models. An additive interaction contrast was used to assess the treatment effects on IgG complexes and C3. For the presence of IgG complexes, the interaction contrast compares the difference in proportions for mice given anti-CTLA-4 and control antibody mice, with the difference in proportions for mice given the combination of anti-CTLA-4 and anti-4-1BB and mice given anti-4-1BB alone. A similar contrast can be calculated for C3. If anti-4-1BB has no diminishing effect on anti-CTLA-4, then the two estimated differences should be the same (null hypothesis).

Results

Combined effect of anti-CTLA-4 and anti-4-1BB antibodies in the induction of CD8 T-cell-mediated tumor rejection. Two models, one of minimal disease and one of large established tumors, were used to test the antitumor effect of combining anti-4-1BB and anti-CTLA-4 mAb treatments. C57BL/6 mice were challenged with a s.c. inoculation of MC38 colon cancer cells, and at different times after tumor cell inoculation, antibodies were injected into tumor-challenged mice and the tumor size and incidence were monitored by physical examination.

In the minimal disease model, the mice were treated with hamster IgG plus rat IgG, anti-4-1BB plus hamster IgG, anti-CTLA-4 plus rat IgG, or anti-4-1BB plus anti-CTLA-4 mAbs starting at 48 hours after inoculation of tumor cells. The antibodies were given i.p. on days 2, 9, and 16. Treatment with either anti-4-1BB or anti-CTLA-4 mAb alone resulted in a delay in tumor growth with one of five mice in each group rejecting tumors, whereas four of five mice treated with both anti-CTLA-4 and anti-4-1BB mAbs were tumor-free at the conclusion of the experiment. Figure 1*A* displays the tumor growth measurements for each mouse. To compare growth rates between groups, a linear random effects model was applied to the data. The combination therapy significantly reduced the daily growth in tumor size by 4.6 mm^2/d over anti-CTLA-4 alone (P = 0.0094). Furthermore, the combination therapy significantly reduced growth by 8.4 mm^2/d over anti-4-1BB alone (P = 0.0006). In addition to the growth rate, the actual tumor sizes were compared between the treatment groups at 6 weeks after the initial tumor challenge. The average tumor size at 6 weeks was significantly smaller for mice given the combination therapy (27.5 mm^2) compared with mice given either anti-CTLA-4 (137.8 mm^2; P = 0.0251) or anti-4-1BB (287.6 mm^2; P = 0.0006) separately. Thus, in the setting of minimal tumor burden, the combination of anti-4-1BB and anti-CTLA-4 mAbs results in significant delays in tumor growth over anti-4-1BB or anti-CTLA-4 given separately.

To determine if the antitumor effects of combination mAb treatment against small tumor burden could be extended to therapeutic applications against larger tumor burdens, mice with established tumors were treated with antibodies. Wild-type C57BL/6 mice were challenged with a s.c. inoculation of MC38 colon cancer cells. Tumors were allowed to grow for 14 days, at which point mice with established tumors (usually >7 mm in diameter) were selected and divided randomly into four treatment groups: hamster IgG plus rat IgG, anti-4-1BB plus hamster IgG, anti-CTLA-4 plus rat IgG, and anti-4-1BB mAb plus anti-CTLA-4 mAb. The antibodies were given i.p. on days 14, 21, and 28 after tumor challenge. As shown in Fig. 1*B*, treatment with anti-CTLA-4 mAb did not impede tumor growth when compared with control IgG treatment, although rejection was seen in one of the eight mice in the group. Treatment with anti-4-1BB mAb slowed tumor growth somewhat, but only one in eight mice rejected the tumor. In contrast, combination therapy with both anti-CTLA-4 and

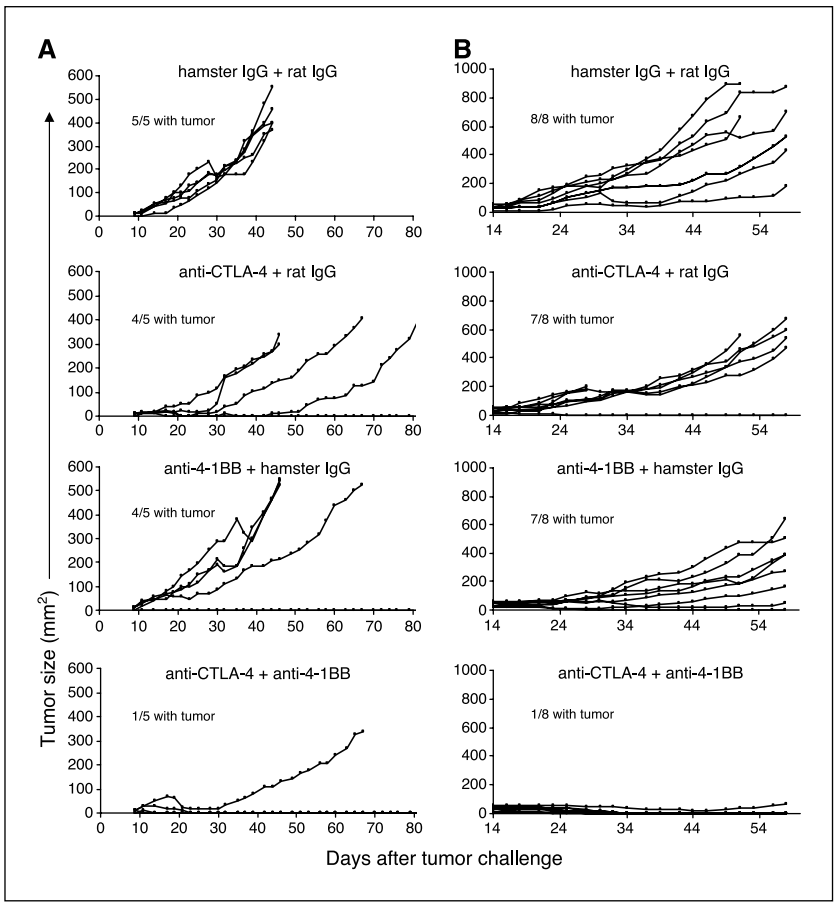

Figure 1. Therapeutic effect of anti-4-1BB and anti-CTLA-4 antibodies in both minimal disease (*A*) and established tumor (*B*) models. *A,* therapy of minimal disease. C57BL/6 mice were inoculated s.c. with 5×10^5 MC38 cells. On days 2, 9, and 16 after tumor cell injection, control hamster and rat IgG, anti-CTLA-4, and/or anti-4-1BB antibodies were injected. Tumor sizes were measured by physical examination. Growth kinetics of tumors, with each line representing tumor growth in one mouse. Sizes are products of long and short diameters of the tumor. *B,* therapy of established tumors. As in (*A*), except that therapy started on day 14 after tumor challenge, all mice had established tumors ranging from 9 to 60 mm^2 in size before treatment with mAbs was started. The combined effect of the two antibodies on established tumors has been repeated thrice.

anti-4-1BB mAbs led to the eradication of tumors in seven of eight mice and prevention of further tumor growth in the remaining mouse. As above, growth rates between groups were compared by applying a linear random effects model to the data. The combination therapy significantly reduced the daily growth in tumor size by 10.6 mm^2/d over anti-CTLA-4 alone ($P < 0.0001$). Furthermore, the combination therapy significantly reduced growth by 6.2 mm^2/d over anti-4-1BB alone ($P = 0.0002$). In addition to the growth rate, the actual tumor sizes were compared between the treatment groups at 8 weeks after the initial tumor challenge. The estimated average tumor size at 8 weeks was significantly smaller for mice given the combination therapy [-1.7 mm^2; 95% confidence interval (95% CI), -10.8 to 7.5 mm^2] compared with mice given either anti-CTLA-4 (404.9 mm^2; 95% CI, 285.4-524.4 mm^2; $P < 0.0001$) or anti-4-1BB (228.4 mm^2; 95% CI, 200.4-689.9 mm^2; $P = 0.0004$) separately. Therefore, the combination mAb also seems to significantly delay tumor growth over anti-CLTA-4 or anti-4-1BB separately in larger tumor burdens as well.

MC38 is known to form liver metastasis (30). To evaluate the effect of therapeutic antibodies on liver metastasis, all mice enrolled in the experiments were analyzed for liver metastasis by histology. As shown in Table 1, ~60% of the control IgG-treated mice had micrometastasis in the liver. Treatments with either anti-CTLA-4 or anti-4-1BB antibodies alone reduced the rate of metastasis somewhat, although the reduction did not reach statistical significance. Remarkably, only 1 of 22 mice in the group treated with both antibodies had liver metastases. Using a logistic regression model, we found that the odds of liver metastasis for mice given anti-4-1BB alone were ~4.7 times higher than the odds for mice given both anti-4-1BB and anti-CTLA-4 (95% CI, 1.6-13.7; $P = 0.0050$). Similarly, the odds of liver metastasis were 3.6 times higher for mice given anti-CTLA-4 only compared with mice given both treatments (95% CI, 1.3-10.2; $P = 0.0174$). Thus, combination therapy significantly reduces liver metastasis by MC38 when compared with treatment with either antibody alone.

To test whether this regimen can be effective for other tumor models, we used the same protocol for B16 melanoma. Our preliminary studies showed that B16F1, a melanoma cell line, failed to respond to the combination therapy (Supplementary Fig. S1).

To determine which subset of immune cells was contributing to the antitumor effect elicited by combination mAb treatment, the major subsets of lymphocytes were depleted with mAbs. MC38 tumor cells were injected s.c. Once tumors were palpable, tumor-bearing mice were separated into four groups. Each group had a series of i.p. antibody injections to deplete differing subsets of immune cells, including no depletion with normal rat IgG, CD4 T-cell depletion with anti-CD4 mAb (GK1.5), CD8 T-cell depletion with anti-CD8 mAb (2.4.3), and natural killer (NK)–cell depletion with anti-NK1.1 mAb (PK136). In addition, all mice in all groups were treated with anti-CTLA-4 plus anti-4-1BB mAbs once weekly for 3 weeks. Adequate depletion of immune cell subsets was evaluated by flow cytometry of peripheral blood taken from mice immediately before completion of the experiment (data not shown). As expected, mice with no depletion of immune cells responded to treatment with anti-CTLA-4 combined with anti-4-1BB mAb (Fig. 2). Similarly, depletion of NK cells and CD4 T cells

Table 1. Combination therapy substantially reduces liver metastases

Group	Treatment	No. (%) mice with metastasis	Group comparison *P*
1 (n = 19)	Hamster IgG + rat IgG	11 (57.8)	
2 (n = 18)	Anti-CTLA-4 + rat IgG	6 (33.3)	0.1383 (vs group 1)
3 (n = 21)	Anti-4-1BB + hamster IgG	8 (38.1)	0.2136 (vs group 1)
4 (n = 22)	Anti-CTLA-4 + anti-4-1BB	1 (4.5)	0.0007 (vs group 1) 0.0174 (vs group 2) 0.0050 (vs group 3)

NOTE: Data are summarized from four independent experiments. At least two sections per liver were examined after H&E staining.

did not affect the antitumor activity of combination anti-CTLA-4 plus anti-4-1BB mAb therapy. The depletion of CD8 T cells, however, abrogated the antitumor activity of combination antibody therapy. At day 28, the estimated average tumor size for mice with depletion of CD8 T cells (92.3 mm^2; 95% CI, 64.5-120.1 mm^2) was significantly higher than the average tumor sizes for mice with no depletion of immune cells (28.7 mm^2; 95% CI, −17.1 to 74.4 mm^2), mice with depleted CD4 T cells (16.7 mm^2; 95% CI, 1.0-32.4 mm^2), and mice with depleted NK cells (9.3 mm^2; 95% CI, −8.3 to 26.9 mm^2). These data show that the tumor-eradicating effect of anti-CTLA-4 and anti-4-1BB mAb treatment is CD8 T-cell dependent.

Combination therapy reduces autoimmune effects. Two approaches were taken to determine whether increased cancer immunity is associated with increased autoimmunity. First, ELISA was used to determine serum anti-dsDNA levels in MC38 tumor-challenged mice 3 weeks after treatment with control IgG, anti-CTLA-4, anti-4-1BB, or combined anti-CTLA-4 plus anti-4-1BB mAbs (Fig. 3*A*). ANOVA done on the data at a serum dilution of 1:150 showed that the anti-dsDNA levels in mice given anti-CTLA-4 were significantly higher than the control IgG-treated mice (P = 0.0006), anti-4-1BB-treated mice (P = 0.0112), and anti-CTLA-4 plus anti-4-1BB–treated mice (P = 0.0214). Based on an interaction contrast, the addition of anti-4-1BB significantly lowered anti-dsDNA production that was seen with anti-CTLA-4 only treatment (P = 0.0032). Furthermore, the estimated anti-dsDNA levels in mice treated with combination anti-CTLA-4 and anti-4-1BB were comparable with levels obtained from those treated with anti-4-1BB alone (P = 0.9822) and hamster IgG plus rat IgG (P = 0.2098). These findings indicate that the addition of anti-4-1BB to anti-CTLA-4 treatment suppresses anti-dsDNA levels to those seen with no anti-CTLA-4 treatment at all.

To confirm the pathologic significance of the anti-DNA antibodies, antibody and complement deposition in the kidneys of tumor-challenged, antibody-treated mice, harvested 3 to 8 weeks after completion of antibody treatment, were next considered. As shown in Fig. 3*B*, mice treated with anti-CTLA-4 mAb alone were found to have deposition of both IgG (70%) and C3 (60%). An interaction contrast confirmed that the addition of anti-4-1BB to anti-CTLA-4 mAb treatment resulted in significant reductions in positive responses for the deposition of both IgG (P = 0.0045) and C3 (P = 0.0010). Despite these pathologic findings, serologic assays of renal function and urine dipstick analysis were unchanged (data not shown). Thus, in agreement with the mouse model of lupus (24, 25), anti-4-1BB antibodies inhibited anti-dsDNA antibody production and reduced the renal pathology.

In the experiments with established tumors, we microscopically analyzed H&E-treated sections from multiple organs for inflammation (Fig. 4*A*). Although small foci of inflammation could be seen in the intestine and the stomach (data not shown), most inflammation was seen in the lung and the liver. Inflammation in the lung was observed in tumor-challenged mice that received control IgG, anti-CTLA-4 mAb, and, to a lesser extent, anti-4-1BB antibody. Inflammation in the lungs of mice treated with combination anti-CTLA-4 plus anti-4-1BB was significantly lower than those treated with anti-CTLA-4 alone (P = 0.0165) and control IgG alone (P = 0.0483). Surprisingly, anti-4-1BB antibody, but not anti-CTLA-4 antibody, greatly enhanced inflammation in the liver as judged by both the number and the size of foci. Importantly, the hepatic inflammation in the anti-4-1BB-treated group was significantly decreased (P = 0.0002) when combined with anti-CTLA-4 (Fig. 4*B*). Thus, combination therapy with both anti-4-1BB and CTLA-4 can enhance antitumor immunity while reducing inflammation to normal host organs. Nevertheless, it should be emphasized that the observed hepatic inflammation was not associated with changes in standard serologic liver function tests (data not shown) or other clinical findings. Moreover, the liver inflammation in the anti-4-1BB-treated group occurs regardless of whether there is a metastasis in the liver (data not shown). Thus, the inflammation is not a secondary consequence of tumor metastasis.

To substantiate the effect of anti-4-1BB antibody on liver inflammation of T cells, mononuclear cells were isolated from control IgG-treated and anti-4-1BB-treated mice and analyzed for their cell surface markers. As shown in Fig. 4*C*, the number of CD4 T cells in the mononuclear cell preparation was not significantly increased by the treatment (P = 0.3951). A significant increase in CD8 T cells was observed after anti-4-1BB treatment (P < 0.0001). Regardless of antibody treatment, the majority of the T cells in the livers were activated as judged by down-regulation of CD62L

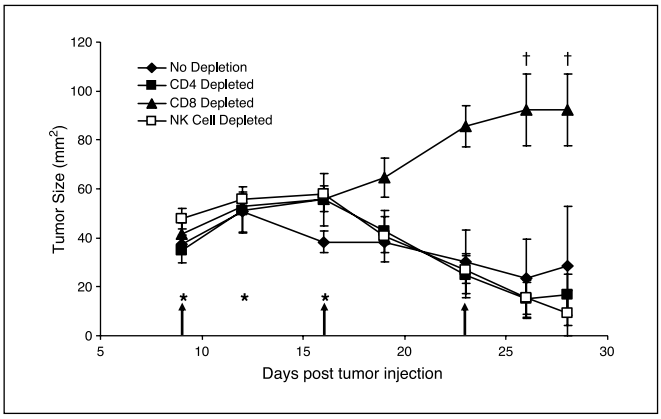

Figure 2. CD8 T cells, but not CD4 or NK cells, are essential for antibody-induced tumor rejection. Tumor-bearing mice were depleted of CD4, CD8, or NK cells by three injections of antibodies specific for either CD4, CD8, or NK1.1 on days 9, 12, and 16 after tumor cell inoculation (*asterisks*). Therapeutic antibodies (anti-CTLA-4 + anti-4-1BB) were injected on days 9, 16, and 23 (*vertical arrows*). *Points,* mean of tumor sizes (n = 3); *bars,* SE. †, P < 0.05, CD8-depleted group compared with each of the other groups.

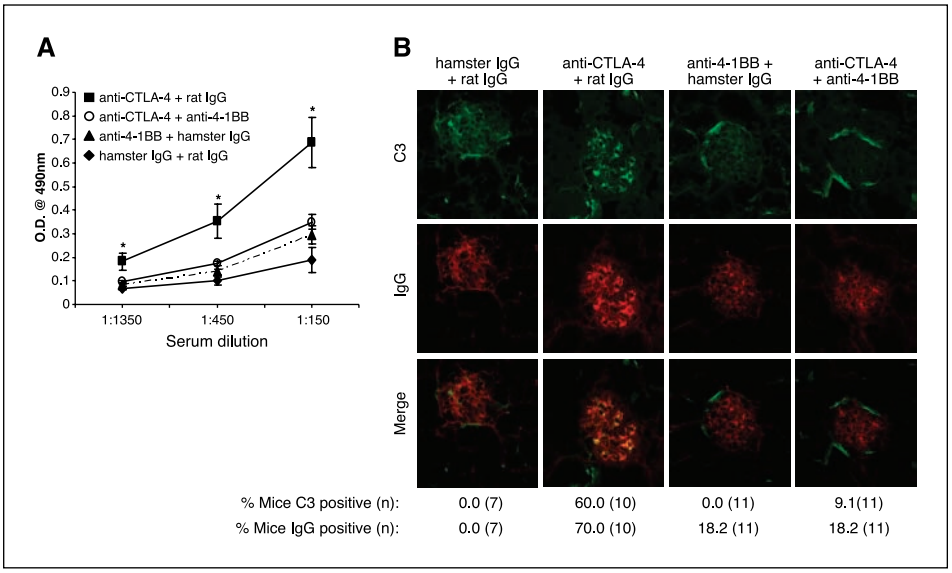

Figure 3. Combination therapy reduced production of anti-DNA antibodies and lupus-like pathology in the kidney. Mice received three weekly antibody treatments with hamster IgG + rat IgG, anti-CTLA-4 + rat IgG, anti-4-1BB + hamster IgG, or anti-CTLA-4 + anti-4-BB mAb after s.c. tumor cell challenge with MC38. *A,* serum anti-dsDNA antibodies collected at 43 days after tumor cell challenge. Representative of three independent experiments. *Points,* mean $A_{490\ nm}$ from groups of five mice; *bars,* SE. *, $P < 0.05$, anti-CTLA-4 treated group compared with each of the other groups; $P > 0.05$, combination mAb-treated group compared with control IgG- and anti-4-1BB-treated groups. *B,* deposition of immune complex in the glomeruli as revealed by C3 (*top*) and IgG (*middle*). *Bottom,* merged images. Data are pooled from three independent experiments. Mice were sacrificed between 21 and 79 days after tumor challenge. Two pathologists independently evaluated slides in a blinded fashion, rating each as a positive or negative response based on positive and negative controls. The addition of anti-4-1BB to anti-CTLA-4 mAb treatment resulted in significant reduction in positive responses for both IgG complexes ($P = 0.0045$) and C3 ($P = 0.0010$).

and/or up-regulation of CD44 (Fig. 4*D*). Interestingly, anti-4-1BB caused a massive increase of $CD44^{lo}CD62L^{lo}$ cells, which were barely detectable in the control IgG-treated group. The functional significance of this novel subset is unclear.

Combination of anti-4-1BB and anti-CTLA-4 increases activity of Treg. Both CTLA-4 and 4-1BB are overexpressed in Treg (31–33). We used flow cytometry to determine the distribution of the 4-1BB and CTLA-4 molecules on Treg. Because CTLA-4 normally resides intracellularly (34), we stimulated spleen cells with anti-CD3 at 37°C in the presence of labeled anti-CTLA-4 antibodies. Excess levels of normal hamster IgG and anti-FcR mAb were added to prevent nonspecific binding. After unbound anti-CTLA-4 antibodies were washed away, biotinylated anti-4-1BB antibodies were added at 4°C. As shown in Fig. 5*A*, 4-1BB and CTLA-4 were both expressed on the surface of Treg after short-term stimulation, although their expression seemed to be independent of each other. Whereas expression of CTLA-4 on the cell surface required stimulation, expression of 4-1BB was constitutive on Treg (data not shown), as others have reported (35).

Expression of 4-1BB and CTLA-4 on Treg raised the intriguing possibility that suppression of autoimmunity by the two antibodies can be achieved by modulating the activity of Treg. To test this possibility, we treated MC38 tumor-challenged mice as described above. Between 2 and 3 weeks after the antibody regimen, the spleen cells were harvested to analyze the number and activity of Treg. As shown in Fig. 5*B*, the proportion of CD4 T cells expressing CD25 was not changed by the antibody treatments. Intracellular staining with fluorochrome-conjugated anti-Foxp3 mAb showed that >90% of these $CD4^+CD25^+$ T cells expressed Foxp3 (Fig. 5*C*). Interestingly, anti-4-1BB and CTLA-4 antibodies drastically increased the Treg activity (Fig. 5*D*). On a cell-to-cell basis, Treg from the double antibody-treated group were 4- to 8-fold more efficient than those isolated from the control IgG-treated group. These results show that combination therapy with anti-CTLA-4 plus anti-4-1BB mAbs increases Treg activity.

Our analysis for T cells specific for gp70, the only known tumor antigen in MC38 tumor cells (36), indicated that the anti-CTLA-4 and/or anti-4-1BB did not increase the percentage of the tumor antigen-specific T cells (Supplementary Fig. SS2).

Anti-4-1BB antibody reduced antibody response to xenogeneic anti-CTLA-4 antibodies. One of the obstacles to repeated antibody therapy is the enhancement of host antibody responses to the therapeutic antibodies (37, 38). Because 4-1BB is known to reduce antibody response to proteins, we evaluated the effect of anti-4-1BB antibodies on host response to anti-CTLA-4 antibodies. As shown in Fig. 6, very little, if any, anti-antibody response was detected in mice treated with either control IgG or 4-1BB. Consistent with the ability of anti-CTLA-4 mAb to facilitate CD4 T-cell responses (39), mice treated with anti-CTLA-4 plus rat IgG developed strong host antibody responses against the given 4F10 and rat IgG (Fig. 6*A* and *B*). This response was reduced by >30-fold when anti-4-1BB was coadministered with anti-CTLA-4 mAb. These data suggest that anti-4-1BB antibodies can potentially increase the duration of other coadministered therapeutic proteins by reducing host responses to the therapeutics.

In human CTLA-4 knock-in mice, a combination of anti-mouse 4-1BB and anti-human CTLA-4 antibodies induced tumor rejection and long-lasting cancer immunity. Because anti-4-1BB reduces the production of antibodies against the anti-CTLA-4 antibodies, an interesting issue is whether the enhancement of tumor rejection by anti-4-1BB is solely due to its effect in suppressing antibody response. We have recently produced a human CTLA-4 gene knock-in mouse in which the mouse CTLA-4 gene is replaced with its human counterpart (28). This mouse allowed us to test if the antitumor effect of the anti-human CTLA-4 antibodies can be enhanced by anti-4-1BB antibody. As shown in

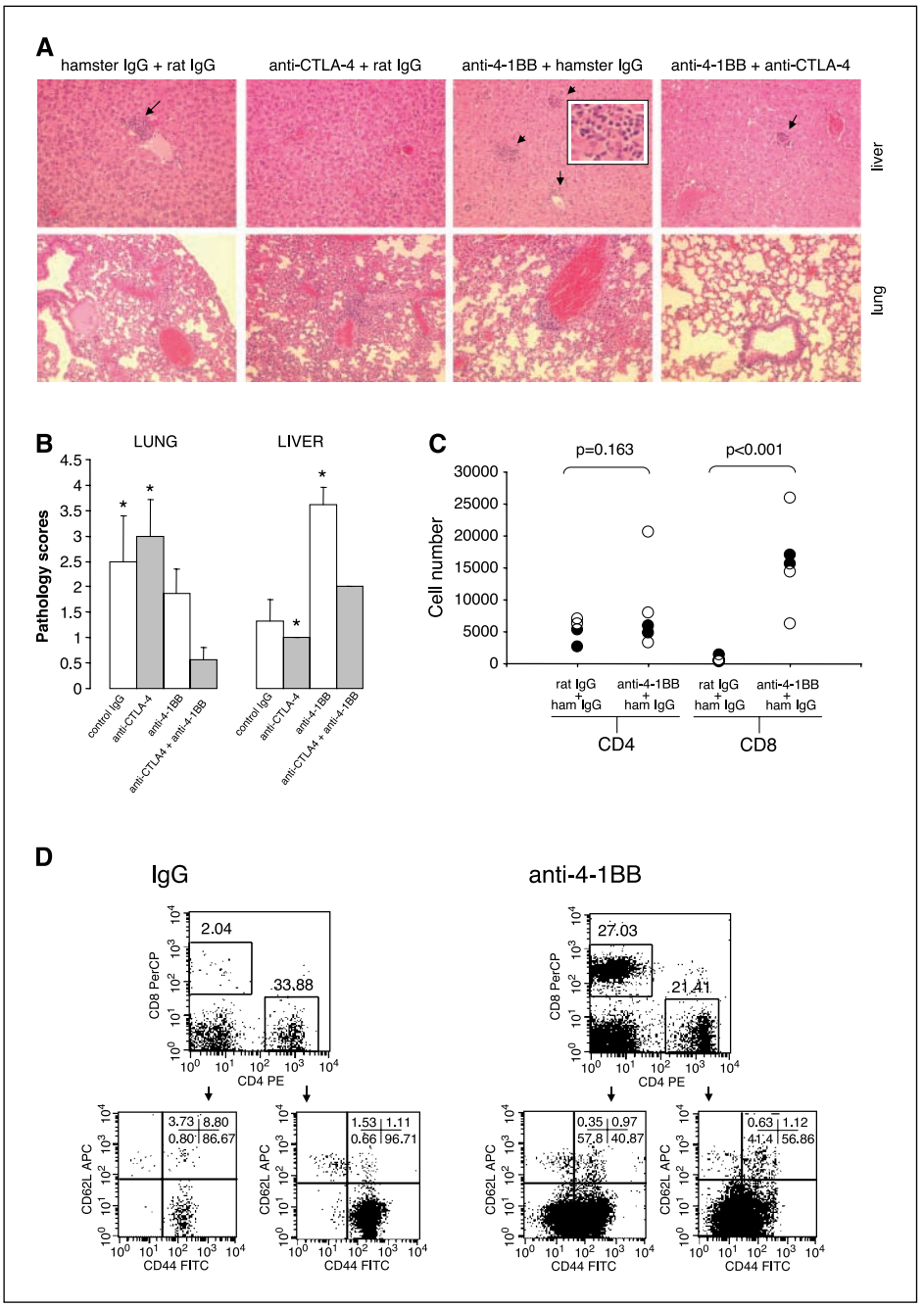

Figure 4. Combination therapy reduced inflammation in the liver and the lung associated with individual anti-4-1BB or anti-CTLA-4 antibody treatment. *A,* H&E staining of liver (*top*) or lung (*bottom*) sections of tumor-bearing mice that received hamster IgG + rat IgG, anti-CTLA-4 + rat IgG, anti-4-1BB + rat IgG, or anti-CTLA-4 + anti-4-1BB antibodies. *B,* summary of pathology scores. *Columns,* mean pathologic scores in liver and lung according to the following criteria; *bars,* SE. Lung: 0, no inflammation; 1, mild inflammation with perivascular lymphocytic infiltration, <10% lung sections involved; 2, mild to intermediate inflammation with increased infiltration of lymphocytes, plasma cells, and interstitial fibrosis and mild consolidation of lung parenchyma, 10% to 25% lung sections involved; 3, intermediate to severe inflammation with increased infiltration of lymphocytes, plasma cells, and some neutrophils and eosinophils, interstitial fibrosis with 30% to 60% lung sections involved; 4, severe acute inflammation with predominant infiltration of neutrophils, pulmonary edema, and consolidation of lung parenchyma, >60% lung sections involved. Liver: 0, no inflammation; 1, mild inflammation with <15 small foci of 5 to 10 lymphocytes around triad, central vein, or parenchyma; 2, mild to intermediate inflammation with <5 medium-sized foci of 10 to 30 lymphocytes around triad, central vein, or parenchyma, or mild fibrosis is present in medium-sized inflammatory foci or >15 small foci of inflammation; 3, intermediate to severe inflammation with large foci of 30 to 70 cells consisting of lymphocytes, neutrophils, and eosinophils; 4, microabscess formation with >100 cells consisting of predominantly neutrophils and eosinophils. *, $P < 0.05$, compared with anti-CTLA-4 + anti-4-1BB–treated group. *C* and *D,* abundance and phenotypes of T cells in the livers of control IgG-treated or anti-4-1BB-treated mice. Groups of C57BL/6 mice ($n = 5$ per group) were challenged with 5×10^5 MC38 cells. Beginning on day 2, the mice received three weekly injections of rat IgG + hamster IgG (100 and 800 μg/injection, respectively) or anti-4-1BB + rat IgG (100 and 800 μg/injection, respectively). Two to three mice from each group were sacrificed on days 41 and 56 and the composition of intrahepatic lymphocytes was analyzed by flow cytometry. Perfused livers were ground with frosted microscope slides and homogenates were passed through a cell strainer and washed. Lymphocytes were isolated by Ficoll and stained with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD44, and anti-CD62L antibodies. *C,* summary of the number of CD4 and CD8 T-cell subsets in the livers of antibody-treated mice. *Open circles,* data from experiment 1; *filled symbols,* data from experiment 2. *D,* phenotype of CD4 and CD8 T cells. Representative fluorescence-activated cell sorting profiles of intrahepatic lymphocytes isolated from antibody-treated mice. Note the dramatic increase in the novel subset of $CD44^{lo}CD62L^{lo}$ after anti-4-1BB treatment.

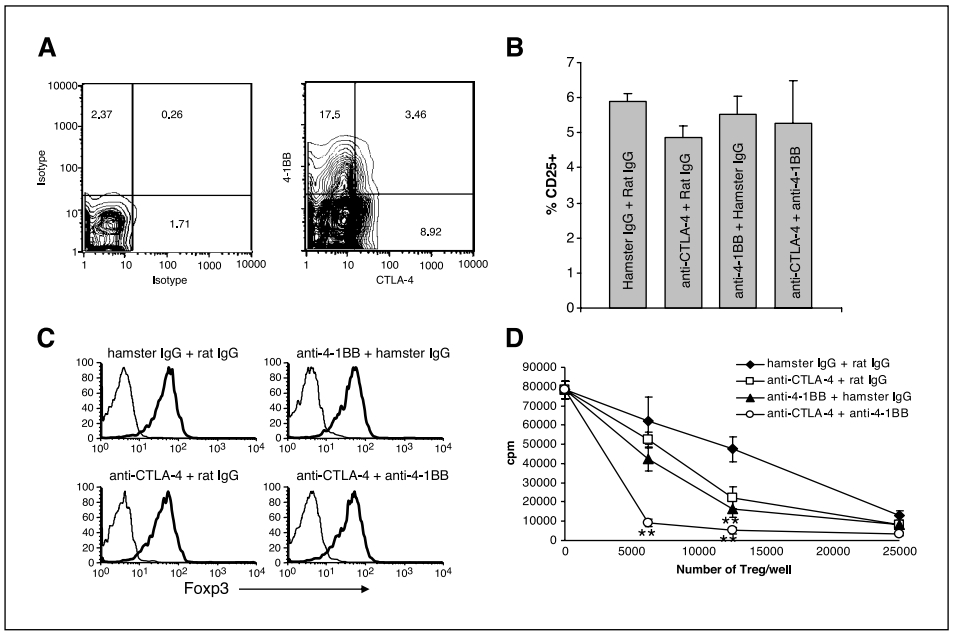

Figure 5. Combination of anti-4-1BB and anti-CTLA-4 antibodies enhanced function of Treg in the mice. *A,* expression of 4-1BB and CTLA-4 on the cell surface of Treg isolated from untreated and unchallenged C57BL/6 mice. Spleen cells were analyzed for expression of CD4, CD25, 4-1BB, and CTLA-4 as described in Materials and Methods. *Right,* a profile for expression of 4-1BB and CTLA-4 on gated $CD4^+CD25^+$ T cells; *left,* a profile for expression of isotype control. *B* and *C,* % $CD25^+$ (*B*) and Foxp3 expression (*C*) of Treg in tumor-challenged mice treated with hamster IgG + rat IgG, anti-CTLA-4 + rat IgG, anti-4-1BB + hamster IgG, or anti-CTLA-4 + anti-4-1BB antibodies. One to 3 weeks after the third antibody injection, the spleens ($n = 3$) were harvested and analyzed for the proportion of $CD4^+CD25^+$ T cells. *Columns,* mean; *bars,* SE (*B*). Individual spleens were also analyzed for intracellular Foxp3 expression (*bold line*) and compared with staining with isotype control (*thin line*; *C*). *D,* treatment with anti-CTLA-4 and anti-4-1BB improved Treg function. Treg were purified from pooled spleen cells from each antibody treatment group by MACS beads and tested for their ability to inhibit proliferation of $CD4^+CD25^-$ spleen cells as described in Materials and Methods. *Points,* mean of triplicate cultures; *bars,* SE. No group difference was seen at the highest cell dose ($P = 0.6209$). **, $P < 0.01$, at the lowest cell dose, the differences between the double antibody treatment and all other groups were highly significant. In comparison with control IgG-treated mice, highly significant differences were also observed in the double antibody-treated and anti-4-1BB-treated groups. The enhanced suppressor activity of the antibody-treated group has been repeated thrice.

Fig. 7*A*, whereas both anti-human CTLA-4 (L3D10) and anti-4-1BB antibody (2A) alone caused delayed tumor growth, a combination of the two antibodies resulted in the most significant tumor rejection. Respectively, in the groups treated with anti-CTLA-4, 4-1BB, or the two antibodies, one of seven, two of seven, or five or seven mice never developed tumors, whereas all mice in the untreated group developed tumors. Because the anti-human CTLA-4 antibody is of mouse origin, the effect of 4-1BB antibody cannot be attributed to its suppression of antibodies to therapeutic anti-CTLA-4 antibodies. Moreover, our data also showed that the superior effect of combination therapy will likely be applicable to anti-human CTLA-4 antibody-based immunotherapy.

To test whether the double antibody-treated mice were immune to further tumor cell challenge, we challenged them with tumor cells at 110 days after their first tumor cell challenge. As shown in Fig. 7*B*, all of the five double antibody-treated mice that had rejected the tumor cells in the first round remained tumor-free, whereas control naive mice had progressive tumor growth. Thus, combination therapy also induced long-lasting immunity to the cancer cells.

Discussion

Combination therapy with anti-4-1BB and anti-CTLA-4 antibodies seems to enhance cancer immunity and reduce measurable autoimmune effects. Our results indicate that cancer immunity and autoimmunity are not necessarily linked. Several lines of recent data, primarily in autoimmune depigmentation associated with

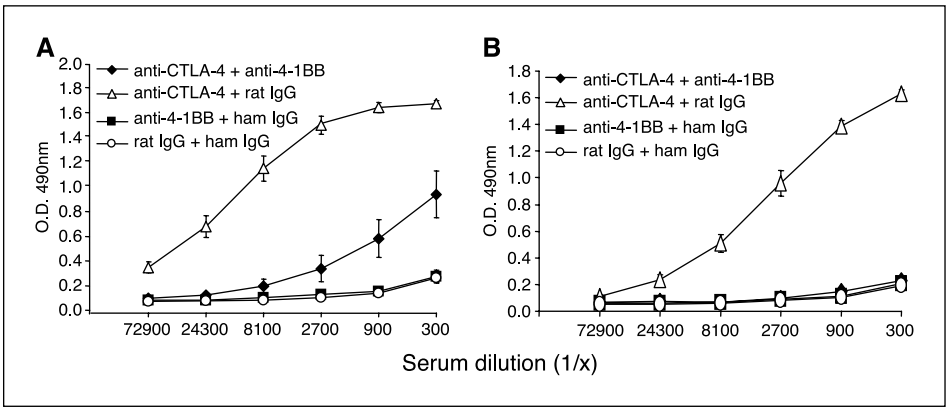

Figure 6. Combination therapy reduced host response to anti-CTLA-4 antibodies. Hamster anti-mouse-CTLA-4 (*A*) or rat anti-mouse-4-1BB (*B*) antibodies were coated in ELISA plates. Different dilutions of sera from groups of five mice each, as those used in Fig. 3*A*, were added to the plates. The relative amounts of antibody bound were determined using a secondary step reagent (biotinylated goat anti-mouse antibodies that were depleted of reactivity to rat and hamster IgG by absorption). *Points,* mean $A_{490\ nm}$; *bars,* SE. Similar reduction of host antibody response to anti-CTLA-4 and 4-1BB was observed when tumor-free mice were treated with the same antibodies (data not shown).

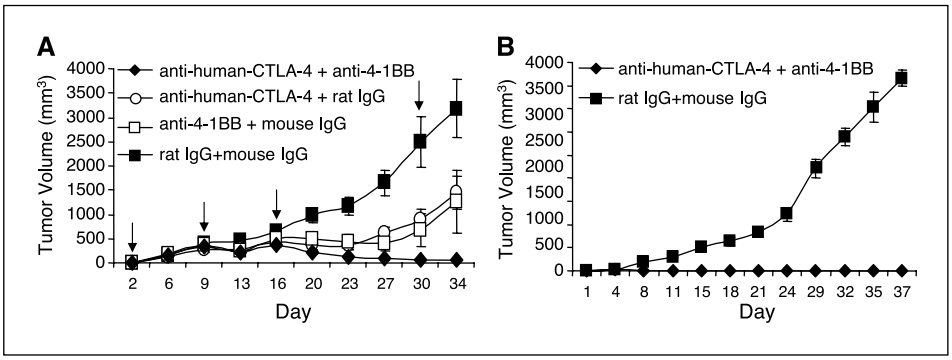

Figure 7. Combination therapy with anti-4-1BB and anti-human CTLA-4 antibody in human CTLA-4 gene knock-in mice. *A,* therapeutic effect. Human CTLA-4 knock-in mice were inoculated with 5×10^5 MC38 tumor cells s.c. Two days later, groups of seven mice were treated with rat and mouse IgG, anti-4-1BB and mouse IgG, anti-human CTLA-4 and rat IgG, or anti-human CTLA-4 and anti-4-1BB (*arrows*). *Points,* mean tumor volume ($n = 7$); *bars,* SE. All treatments significantly reduced tumor growth ($P < 0.001$), and the double antibody treatment group show significantly reduce tumor size in comparison with either control ($P < 0.0001$), anti-CTLA-4 antibody ($P = 0.0007$), or anti-4-1BB antibody treatment ($P = 0.03$). All tumor-bearing mice were sacrificed when the control IgG-treated group reached early removal criteria. *B,* long-lasting immunity in mice that received combination therapy. Tumor-free mice in the double antibody-treated group developed long-lasting immunity to MC38 tumors. At 110 days after the first tumor cell challenge, the double antibody-treated, tumor-free mice or control naive mice were challenged with 5×10^5 tumor cells s.c. Tumor growth was monitored by physical examination. Note that all of the mice that rejected the tumors in the first round were completely resistant to rechallenge, whereas all naive mice had progressive tumor growth.

melanoma antigen, are consistent with this notion. Thus, although antibodies against TYRP-1/gp75 can produce both tumor rejection and autoimmune depigmentation, the autoimmune destruction requires 5-fold more antibodies and can be distinguished from tumor rejection by the requirement for FcR and complement (9, 40). Likewise, after immunization with antigen TYRP-2/DCT, T-cell-mediated tumor rejection can be perforin independent, whereas autoimmune depigmentation requires perforin (41, 42). Whereas these studies have raised theoretical possibilities to unravel cancer immunity and autoimmunity, our studies provide a novel and generally applicable approach to enhance cancer immunity and reduce autoimmunity.

An interesting issue is the immunologic basis by which combination therapy with anti-CTLA-4 and anti-4-1BB mAbs uncouples tumor immunity and autoimmunity. Because the two antibodies enhance tumor immunity by mechanisms unknown to overlap, it is plausible that a combination of the two may lead to at least an additive effect as shown here. Based on statistical analysis, the combination therapy could possibly have more than an additive effect in tumor rejection. The surprising observation that, when used in combination, the two antibodies reduce each other's side effects is not fully explained at this stage. The reduction of serum anti-dsDNA antibody and renal deposition of immune complexes by anti-4-1BB antibodies has been reported in two lupus models presumably by suppressing CD4 T-cell responses (24, 25). We showed here that a combination of the two antibodies substantially increased the *in vitro* suppressor function of Treg. Given the potent effect of Treg in modulating autoimmune diseases (43–46), it is plausible that the suppression of autoimmunity is based on the increased Treg activity in doubly treated mice. What remains unexplained is why cancer immunity, which is also hindered by Treg (47), was promoted by the treatment that increased Treg activity. We believe this paradox can be reconciled either by positing a robust cancer immunity that overcomes the increased Treg activity or by hypothesizing that the Treg that restrains cancer immunity differ from those that modulates autoimmunity. Additional studies are needed to resolve this important issue.

Anti-CTLA-4 antibodies have been shown to enhance autoimmune diseases in several animal models (19–22). More recently, it has also been reported to induce autoimmune disease in cancer patients (23). Here, it has been shown that in cancer-bearing mice this antibody increased the production of anti-DNA antibodies and deposition of immune complexes in the kidney. Moreover, these side effects were controlled by coinjection of anti-4-1BB antibody. The effect of anti-4-1BB antibodies in kidney pathology can be due either to suppression of antixenogeneic IgG and/or autoantibodies. In other studies, anti-4-1BB antibody suppressed autoimmune diseases in at least two models (24, 25), but immunotherapy with anti-4-1BB antibody was not totally devoid of side effects. For example, anti-4-1BB mAb was found to induce liver inflammation in the lupus-prone mice.[4] Similarly, our results indicate that anti-4-1BB antibody also increased inflammation in the liver. There is, however, no widespread liver damage as serum liver function tests were within normal limits (data not shown). These data suggest that side effects to different organs can be differentially modulated. Surprisingly, such inflammation can be suppressed by coinjection with anti-CTLA-4 antibody. Although the mechanism of the mutual antagonism of the two antibodies is unclear, such an antagonism in autoimmunity and additive effect in cancer rejection suggests that the combination may be of general significance for cancer therapy.

Finally, one of the obstacles to protein-based immunotherapy is host immunity to the therapeutic proteins. In the case of antibodies, the host can mount antibodies to xenotypic, allotypic, and idiotypic epitopes (37, 38). The xenotypic response can be eliminated by complete humanization, although other anti-antibody responses require special considerations. The obstacle is more obvious for anti-CTLA-4 antibody as it is an adjuvant in itself. Previous work by Mittler et al. showed a significant suppression of T-cell-dependent humoral immune response (48). Our data show that coadministration of anti-4-1BB antibodies reduces host responses to the anti-CTLA-4 antibody, which suggests another advantage of combination therapy using anti-CTLA-4 and anti-4-1BB antibodies.

Taken together, our data show that combination therapy with anti-CTLA-4 and anti-4-1BB antibodies offers three major advantages (i.e., an increased effect in cancer immunity, mutual suppression of autoimmune side effects, and amelioration of anti-antibody

[4] Y. Fu, personal communication.

responses). It is of great interest to test the general applicability of this approach in therapy of additional mouse cancer models and in cancer patients. An important, but largely unaddressed, issue is whether the two effects can be uncoupled in models in which the same antigens are involved in tumor rejection and autoimmune side effects (5, 49). Earlier work (9, 40–42) showed distinct effector mechanisms in tumor rejection and autoimmune destruction, which suggested that the uncoupling is theoretically possible. Clearly, additional studies are needed to establish the scope of applicability of combination therapy described herein. Nevertheless, the fact that some autoimmune side effects can be uncoupled from tumor immunity is of general significance for cancer immunotherapy.

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Combination Therapy with Anti–CTL Antigen-4 and Anti-4-1BB Antibodies Enhances Cancer Immunity and Reduces Autoimmunity

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