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4-1BB-Specific Monoclonal Antibody Promotes the Generation of Tumor-Specific Immune Responses by Direct Activation of CD8 T Cells in a CD40-Dependent Manner

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4-1BB (CD137) is a member of the TNFR superfamily (TNFRSF9). T cell expression of 4-1BB is restricted to activated cells, and cross-linking has been shown to deliver a costimulatory signal. Here we have shown that treatment of tumor-bearing mice with agonistic 4-1BB-specific Abs can lead to T cell-mediated tumor rejection. In vivo mAb depletion experiments demonstrated that this rejection requires CD8⁺ cells but not CD4⁺ or NK cells. Both IFN-γ and CD40-mediated signals were also required, because no benefit was observed on treatment with 4-1BB mAb in mice in which the genes for these molecules had been knocked out. Interestingly, 4-1BB-mediated stimulation of immune responses in CD40L−/− mice is effective (although at a reduced level), and may suggest the existence of an alternative ligand for CD40. Additional experiments in IL-15−/− mice indicate that IL-15 is not required for either the generation of the primary tumor-specific immune response or the maintenance of the memory immune response. In contrast, the presence of CD4 cells during the primary immune response appears to play a significant role in the maintenance of effective antitumor memory. Finally, in mice in which the number of dendritic cells had been expanded by Fms-like tyrosine kinase3 ligand treatment, the antitumor effects of 4-1BB ligation were enhanced. The Journal of Immunology, 2002, 169: 1792–1800.

M any tumors grow progressively in immunocompetent mice, indicating a failure in the generation of effective immune responses to tumor Ags in these hosts. Mechanisms for the failure to induce protective immunity include production of immunosuppressive substances by the tumor, down-regulation of MHC molecules on tumor cells, or insufficient activation and expansion of specifically reactive subsets of T cells (1–3). However, immunity can be generated against these same tumors using a variety of preimmunization strategies such as simple implantation/excision strategies (4–6). These results demonstrate that priming of tumor-reactive T cells occurs in normal, unmanipulated mice and imply that in many cases tumor growth may be due to inefficient or incomplete activation of effector T cells (7–11).

Studies in a variety of systems have demonstrated that engagement of the TCR alone is not sufficient for full T cell activation, expansion, and cytokine secretion. Additional costimulatory signals are also required, of which the signal delivered by B7 molecules on APCs to CD28 on the T cell is the most widely characterized (12–14). T cells typically receive this costimulatory signal while they are exposed to Ags in the context of MHC by APCs (12, 15–17). Failure to receive a second signal can lead to anergy or apoptosis (18–22). One approach to increase the costimulation of T cells in tumor-bearing mice has been to block or neutralize CTLA-4 (a naturally occurring inhibitor of CD28/B7 interactions), thereby increasing the CD28/B7 interaction (9, 10, 23–25). Alternatively, tumor cells have been directly transfected with B7 family members in an effort to promote their ability to act directly as APC (13, 26, 27). Other strategies include expansion and activation of specific subsets of “professional” APCs (i.e., dendritic cells (DC))² in vivo by treatments of Fms-like tyrosine kinase3 ligand (Flt3L) either alone or in combination with CD40 ligand (CD40L), transfection of tumors with cytokines such as GM-CSF, and ex vivo expansion and Ag loading of DC with defined tumor Ags (7, 28–31). These strategies have in common the goal of increasing APC function by increasing the number of available APC and/or enhancing the Ag-presenting function of the APC. Reciprocal approaches (e.g., enhancing the receptivity of the T cell to signals from the APC or the ability of T cells to proliferate in response to Ag using cytokines such as IL-2, IFN-γ, etc.) have also been tried but have met with only limited success (32, 33). This may be due, in part, to the extremely short half-life and lack of selectivity of these cytokines in vivo.

An alternative approach is to specifically target activated subpopulations of T cells with appropriate costimulatory signals. In this regard, 4-1BB, a member of the TNFR superfamily, represents an attractive potential target. It is expressed on activated, but not resting, CD4 and CD8 T cells, and ligation of this receptor by either its ligand or agonistic Abs has been shown to provide a potent costimulatory signal (34, 35). The costimulatory signal has been shown to be more potent for CD8 T cells than CD4 T cells (35, 36).

Previous studies have shown that administration of agonistic 4-1BB Abs or 4-1BB ligand gene expression by tumors can lead to generation of effective antitumor responses (37–40). Interestingly, preliminary results from our own studies were at odds with those of others that indicated that both CD4 and NK cells were absolutely required for the generation of effective tumor immune responses. In contrast, our results demonstrated that tumor rejection could be achieved in the absence of CD4 and CD8 T cells. This finding suggests that the direct ligation of 4-1BB on tumor cells by agonistic Abs may provide a novel approach for the treatment of tumors expressing 4-1BB.

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2 Abbreviations used in this paper: DC, dendritic cells; CD40L, CD40 ligand; Flt3L, Fms-like tyrosine kinase3 ligand; MSA, mouse serum albumin.

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responses in vivo by agonistic 4-1BB mAb (39). Thus, the studies reported here were undertaken to more fully characterize the cellular and cytokine requirements for the enhancement of tumor immune responses mediated by ligation of 4-1BB.

**Materials and Methods**

**Mice**

Female C57BL/10J, C57BL/6Ncr-TNFtgSmyKik (CD40−/−) and C57BL/6-Lgo−/− (IFN−γ−/−) mice were purchased from Dr. Kripke (University of Texas M. D. Anderson Medical Center, Houston, TX). RENCA renal cell carcinoma was a gift from Dr. C. Tannenbaum (Cleveland Clinic Foundation, Cleveland, OH). K1735 melanoma was a gift from Dr. M. Kripke (University of Texas M. D. Anderson Medical Center, Houston, TX). P815 mastocytoma and B16-F0 melanoma were purchased from ATCC (Manassas, VA). C3H/HeN (MTV−/−) mice were purchased from American Type Culture Collection (ATCC; Manassas, VA). Female (C57BL/6 × DBA/2)F1, B6D2F1, and C57BL/6 Abb/B2 (MHC class II−/−) mice were purchased from TacGen (Germantown, NY). CD40L−/− and IL-15−/− mice were generated at Immunex (Seattle, WA) by homologous gene disruption (41). All mice were age matched at the beginning of each experiment (8–12 wk of age) and kept under specific pathogen-free conditions at Immunex.

**Tumor cell lines**

The B10.2 fibrosarcoma is a methylcholanthrene-induced tumor of C57BL/10 origin, and the 87 fibrosarcoma is a UVB-induced tumor of C3H/HeN (MTV−/−) origin. P815 mastocytoma and B16-F0 melanoma were purchased from American Type Culture Collection (ATCC; Manassas, VA). K1735 melanoma was a gift from Dr. C. Tannenbaum (Cleveland Clinic Foundation, Cleveland, OH). Tumor inoculations were performed by s.c. injection of tumor cell lines purchased from ATCC. Asialo-GM1 antiserum was purchased from Wako Pure Chemical Industries (Osaka, Japan) and used in accordance with the manufacturer’s recommendations.

**Abs and cytokines**

Anti-4-1BB (M6, rat IgG2a) and anti-CD40L (M158, rat IgG2b) mAb were produced and purified at Immunex. Flt3L was produced at Immunex as described previously (28). Purified rat IgG and mouse serum albumin (MSA) were purchased from Sigma-Aldrich (St. Louis, MO). Depleting anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (2.43, rat IgG2b) and anti-NK1.1 (PK136, mouse IgG2a) were purified from culture supernatants of hybridoma lines purchased from ATCC. Asialo-GM1 antiserum was purchased from Wako Pure Chemical Industries (Osaka, Japan) and used in accordance with the manufacturer’s recommendations.

**Statistics**

Biostatistical evaluation of tumor rejection frequencies was conducted by analysis of combined results from multiple independent experiments using Fisher’s exact test. All tests were two-sided and a nominal p value of 0.05 was used to determine statistical significance.

**Results**

**Efficacy of 4-1BB agonism in various experimental tumor model systems**

Previous studies have demonstrated that treatment of tumor-bearing mice with certain mAb specific for murine 4-1BB could promote the generation of effective tumor immunity in vivo (38). To test whether a 4-1BB mAb produced at Immunex (4-1BB (M6)) could similarly act to induce tumor rejection, C57BL/10 mice were injected s.c. with B10.2 fibrosarcoma cells and subsequently treated with two 100-μg i.p. injections of 4-1BB (M6) at various times post-tumor challenge. Fig. 1 shows the results from two experiments in which mice were treated beginning as early as 3 days, or as late as 24 days, after tumor implantation. Treatments at all time points resulted in complete tumor rejection in a high proportion of mice and significant reduction in tumor size and growth rates in the remainder. Treatment of well-established B10.2 tumors beginning as late as day 24 post-tumor challenge resulted in complete tumor rejection of tumors in up to 80% of the tumor-bearing mice treated with agonistic 4-1BB (M6) mAb. A, C57BL/10 mice were challenged s.c. with 5 × 103 B10.2 tumor cells on day 0. Mice were left untreated (n = 10; ○) or were treated with 4-1BB (M6) (150 μg i.p./injection) on days 3 and 6 (n = 10; ◊) or on days 13 and 16 (n = 10; □). B, C57BL/10 mice were challenged with 5 × 105 B10.2 tumor cells on day 0 and treated with rat IgG (100 μg i.p./injection) on days 14, 17, 24, and 27 (n = 10; ○), 4-1BB (M6) (150 μg i.p./injection) on days 14 and 17 (n = 10; ◊), and on days 24 and 27 (n = 9; □). The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (×100).
mice. Tumor rejection induced by treatment with 4-1BB (M6) was mediated by CD8+ T cells and resulted in the generation of long-lasting tumor-specific memory (data not shown).

To determine whether the beneficial effects of 4-1BB (M6) mAb treatment were generalizable to a variety of tumors, a total of six tumor models were tested. 4-1BB (M6) enhanced the rejection of 4 of 6 tumor cell lines tested (Table I). The two tumors not affected by the 4-1BB (M6) treatment (K1735 and B16-F0) were MHC class I negative by flow cytometry (data not shown).

Neither CD4 nor NK cells are required for 4-1BB (M6) enhancement of antitumor immunity

Depletion of CD8 T cells in mice before initiating treatment with 4-1BB(M6) mAb completely abrogated tumor rejection of the

Table I. Beneficial effect of 4-1BB (M6) mAb treatment in promoting tumor rejection in various tumor models

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of Expts.</th>
<th>Control IgG</th>
<th>4-1BB (M6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TBA/totala</td>
<td>% Tumor rejection</td>
</tr>
<tr>
<td>P815</td>
<td>5</td>
<td>41/45</td>
<td>8.9</td>
</tr>
<tr>
<td>B10.2</td>
<td>8</td>
<td>74/74</td>
<td>0</td>
</tr>
<tr>
<td>87</td>
<td>2</td>
<td>15/15</td>
<td>0</td>
</tr>
<tr>
<td>B16-F0</td>
<td>1</td>
<td>10/10</td>
<td>0</td>
</tr>
<tr>
<td>K1735P</td>
<td>1</td>
<td>10/10</td>
<td>0</td>
</tr>
<tr>
<td>RENCA</td>
<td>2</td>
<td>20/20</td>
<td>0</td>
</tr>
</tbody>
</table>

a Mice were challenged with tumor cells (as indicated) by s.c. injection. Groups of mice were treated by i.p. injection of either 4-1BB (M6) or rat IgG Ab control.

b Number of tumor-bearing animals/total number challenged at the termination of the experiment.

FIGURE 2. 4-1BB(M6)-mediated enhancement of tumor immune responses in vivo requires CD8 T cells but are CD4 and NK cell independent. C57BL/10 mice were treated 2 days before and day 5 after tumor challenge with 500 μg of i.p. with depleting Abs to CD4, CD8, or NK1.1 or 100 μl of a 1/5 dilution of anti-asialo-GM1. C57BL/10 mice were challenged s.c. with 5 × 10^5 B10.2 tumor cells on day 0. Blood samples from each group were collected from the saphenous vein, on experimental day 16 after tumor challenge, and analyzed by flow cytometry for CD3, CD4, CD8, and NK cells. Control or Ab-depleted groups were divided into treatments of rat IgG (□) or 4-1BB (M6) (●) (100 μg i.p./injection) on days 5, 8, 11, and 14. Tumor incidence plots are composite results from 2 independent experiments with a total of 20 mice/group.
B10.2 fibrosarcoma in C57B10 mice (Fig. 2). In contrast, depletion of NK cells with either anti-NK1.1 or asialo-GM1 had no effect on rejection of the B10.2 tumors, and neither did depletion of CD4 cells with GK1.5. These results contrast with initial studies reported by Melero et al. (38, 39). Potential differences in results due to the use of different tumors (B10.2 vs P815) in different strains of mice (C57BL/10 vs B6D2F1) were ruled out in a separate experiment in which B6D2F1 mice were depleted of NK, CD4, or CD8 cells using Abs described above; challenged with P815 tumor cells; and treated with 4-1BB (M6) mAb beginning on day 5. Again only CD8 cells were required for tumor rejection subsequent to treatment with 4-1BB (M6) mAb (data not shown). In additional studies by Melero et al. (42) in which P815 cells were transformed to express 4-1BB ligand, the CD4 requirement was lost.

Memory immune responses are induced in 4-1BB (M6)-treated mice

The majority of mice that rejected their initial tumor challenge subsequent to 4-1BB (M6) treatment also rejected the tumor on rechallenge 3 mo later. In mice that had been depleted of NK cells before initial tumor challenge by treatment with either asialo-GM1-or NK1.1-specific mAb, the rate of rejection of the secondary tumor challenge proceeded at a rate and frequency (100%) comparable with those observed in the positive control (i.e., tumor-immune) group. However, the rate and frequency of rejection of the secondary tumor challenge in mice that had been depleted of CD4 T cells during the primary immune response were decreased compared with the other treatment groups (Fig. 3). These data suggest that although CD4 T cells are not required to generate an effective primary effector response to tumors in vivo, they may play an important role in the generation and/or maintenance of memory effector cells.

Tumor rejection in class II−/−, IL-15−/−, and IFN-γ−/− mice

To further investigate the requirements for either CD4 or NK cells in the generation of tumor-immune responses induced by 4-1BB mAb treatment, we used MHC class II gene knockout mice that lacked functional CD4 T cell activity and IL-15 knockout mice, which are deficient in NK cells. Class II−/− mice lacking Ag-specific CD4 T cell function were challenged with the B10.2 tumor cells followed by treatment with 4-1BB (M6). Although these mice were capable of rejecting the primary tumor challenge after 4-1BB (M6) mAb treatment (Fig. 4A), they exhibited a reduction in memory function similar to that seen in the CD4-depleted mice (Fig. 4B).

IL-15 knockout mice have been shown to have a defect in NK cell development (and thus lack mature NK cells) as well as a decrease in CD8+ cells expressing a memory phenotype (41, 43). To assess the role of NK cells in the absence of Ab depletions, IL-15−/− mice were challenged with B10.2 tumor cells and treated with 4-1BB (M6) mAb. Mice deficient in IL-15 rejected the tumor challenge when treated with 4-1BB (M6) mAb (Fig. 5A). Rechallenge of both wild-type and IL-15−/− mice that had successfully rejected the initial tumor implants 3 mo earlier led to tumor rejection with essentially identical kinetics, indicating that IL-15 is not required for maintenance of tumor-reactive memory cells in vivo (Fig. 5B).

We also examined the ability of IFN-γ−/− mice to reject B10.2 tumors with or without 4-1BB (M6) treatment. None of the IFN-γ−/− mice rejected the tumor regardless of 4-1BB (M6) treatment, and the tumors grew more rapidly in the IFN-γ−/− than in the wild-type tumor-bearing animals (Fig. 6). These data indicate that IFN-γ is absolutely required for the generation of effective tumor-immune responses in vivo and that this requirement is not alleviated by treatment with 4-1BB specific mAb.

![FIGURE 3](image-url)  
Antitumor memory responses in mice that had rejected a B10.2 tumor challenge after depleting Abs to CD4, CD8, or NK1.1 and treatments with 4-1BB M6. Mice were allowed to age for 3 mo after primary tumor challenge. Naive mice (●) or mice treated with 4-1BB (M6) (●), NK1.1-depleted (○), asialo-GM1-depleted (□), or CD4-depleted (▲) Abs that have rejected prior tumors were then rechallenged s.c. with 5 × 10^5 B10.2 tumor cells. The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (×100).

![FIGURE 4](image-url)  
B10.2 tumor growth and incidence in wild-type and class II−/− mice treated with agonistic 4-1BB (M6) mAb. A, Wild-type and MHC class II−/− mice were challenged s.c. with 5 × 10^5 B10.2 tumor cells on day 0. Wild-type mice were treated (n = 10/group) with rat IgG (●) or 4-1BB (M6) (■) at 100 μg i.p./injection on days 5, 8, 11, and 14. B, Mice that had rejected an initial B10.2 tumor challenge were allowed to age for 3 months followed by rechallenge with B10.2 tumor cells (5 × 10^3) injected s.c. (●), naive wild-type mice; ■, wild-type 4-1BB (M6)-treated tumor rejectors; ○, MHC class II−/− 4-1BB (M6) rejectors; n = 8/group. The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (×100).
Wild-type mice were treated with rat IgG (\( \text{F}_1 \)) or 4-1BB (M6) (\( \text{F}_2 \)) at 100 \( \mu \text{g} \) i.p./injection on days 5, 8, 11, and 14. IL-15 \( \text{F}_1 \) (10 \( \mu \text{g} \) i.p./injection) on days 5, 8, 11, and 14. Two additional groups of mice (\( \text{n} = 10 \)/group) were treated with rat IgG (\( \text{C}_1 \)) or 4-1BB (M6) (\( \text{C}_2 \)), 100 \( \mu \text{g} \) i.p./injection on days 5, 8, 11, and 14. 8. Mice that had rejected an initial B10.2 tumor challenge were allowed to age for 3 months followed by rechallenge with B10.2 tumor cells (\( 5 \times 10^5 \)) injected s.c. (\( \text{D}_1 \), naive wild-type mice; \( \text{D}_2 \), wild-type 4-1BB (M6); \( \text{D}_3 \), 4-1BB (M6)-treated rejectors). The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (\( \times 100 \)).

**Role of CD40-CD40 interactions in tumor rejection**

Two approaches were used to determine whether CD40-CD40L interactions were required for 4-1BB (M6)-enhanced tumor rejection. The first experiments were conducted with anti-CD40L neutralizing Abs. Administration of anti-CD40L (M158) was initiated on the day of tumor challenge (day 0) and continued throughout the period of treatment with anti-4-1BB (M6). Treatment of tumor-bearing mice with 4-1BB (M6) mAb alone resulted in complete tumor rejection in 90% of the mice. However, in the presence of CD40L, blockade treatment with 4-1BB (M6) mAb resulted in only 10% tumor rejection (Fig. 7A). In the second approach, we used mice in which either CD40 or CD40L had been selectively disrupted. These mice were challenged with the B10.2 fibrosarcoma and treated with 4-1BB (M6) mAb. Mice deficient in CD40 failed to reject the tumor challenge. In contrast, mice deficient in CD40L were able to reject the B10.2 tumor challenge, albeit at a slightly reduced rate compared with 4-1BB (M6)-treated wild-type mice (Fig. 7B). These data may suggest the existence of a second ligand for CD40 that becomes induced in mice on which immune systems develop in the absence of the primary ligand for CD40.

**Synergistic effects of combining 4-1BB mAb with Flt3L treatments**

DCs are potent stimulators in the primary activation of T cells. Recent studies have demonstrated that expansion of DCs in vivo by treatment with Flt3L is also capable of augmenting the generation of T cell-mediated immune responses to tumors (28). In addition, the combination of Flt3L with another costimulatory TNF

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**FIGURE 5.** B10.2 tumor growth and incidence in wild-type and IL-15 \( ^{-/-} \) mice treated with agonistic 4-1BB (M6). A. Wild-type and IL-15 \( ^{-/-} \) mice were challenged s.c. with \( 5 \times 10^3 \) B10.2 tumor cells on day 0. Wild-type mice (\( \text{n} = 10 \)/group) were treated with rat IgG (\( \text{E}_1 \)) or 4-1BB (M6) (\( \text{E}_2 \)) at 100 \( \mu \text{g} \) i.p./injection on days 5, 8, 11, and 14. IL-15 \( ^{-/-} \) mice were treated with rat IgG (\( \text{n} = 5 ; \text{E}_3 \)) or with 4-1BB (M6) (\( \text{n} = 7 ; \text{E}_4 \)), 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. B. Mice that had rejected an initial B10.2 tumor challenge were allowed to age for 3 months followed by rechallenge with B10.2 tumor cells (\( 5 \times 10^5 \)) injected s.c. (\( \text{F}_1 \), naive wild-type mice; \( \text{F}_2 \), wild-type 4-1BB (M6); \( \text{F}_3 \), 4-1BB (M6)-treated rejectors). The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (\( \times 100 \)).

**FIGURE 6.** B10.2 tumor growth and incidence in wild-type and IFN-\( \gamma ^{-/-} \) mice treated with agonistic 4-1BB (M6). Wild-type and IFN-\( \gamma ^{-/-} \) mice (\( \text{n} = 10 \)/group) were challenged s.c. with \( 5 \times 10^3 \) B10.2 tumor cells on day 0. Wild-type mice were treated with rat IgG (\( \text{G}_1 \)) or 4-1BB (M6) (\( \text{G}_2 \)), 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. IFN-\( \gamma ^{-/-} \) mice treated with rat IgG (\( \text{H}_1 \)) or 4-1BB (M6) (\( \text{H}_2 \)), 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (\( \times 100 \)).

**FIGURE 7.** Roles of CD40 and CD40 ligand in tumor growth and incidence in mice treated with agonistic 4-1BB (M6) mAb. A. Wild-type mice (\( \text{n} = 10 \)/group) were challenged s.c. with \( 5 \times 10^3 \) B10.2 tumor cells on day 0, followed by treatment with rat IgG (\( \text{I}_1 \)) or 4-1BB (M6) (\( \text{I}_2 \)), 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. Two additional groups of mice (\( \text{n} = 10 \)/group) were treated from day −1 to day 14 with 150 \( \mu \text{g} \) of anti-CD40L/day and challenged s.c. with \( 5 \times 10^3 \) B10.2 tumor cells on day 0, followed by treatment with rat IgG (\( \text{J}_1 \)) or 4-1BB (M6) (\( \text{J}_2 \)), 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. B. Wild-type, CD40 \( ^{-/-} \), and CD40L \( ^{-/-} \) mice were challenged s.c. with \( 5 \times 10^3 \) B10.2 tumor cells on day 0. Wild-type mice were treated with rat IgG (\( \text{K}_1 \)) or 4-1BB (M6) (\( \text{K}_2 \)) (\( \text{n} = 20 ; \text{K}_3 \)) (100 \( \mu \text{g} \) IP/injection) on days 5, 8, 11, and 14. CD40 \( ^{-/-} \) treated with rat IgG (\( \text{n} = 6 ; \text{K}_4 \)) or with 4-1BB M6 (\( \text{n} = 12 ; \text{K}_5 \)) at 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. CD40L \( ^{-/-} \) treated with rat IgG (\( \text{n} = 6 ; \text{K}_6 \)) or with 4-1BB (M6) (\( \text{n} = 12 ; \text{K}_7 \)), 100 \( \mu \text{g} \) i.p./injection, on days 5, 8, 11, and 14. The tumor size represents the average of only tumor-bearing mice, and tumor incidence is calculated as the number of tumor-bearing over the total number of mice challenged (\( \times 100 \)).
family member (CD40L) yields cooperative effects (29). To determine whether combined treatments of Flt3L and 4-1BB (M6) would result in enhanced tumor rejection, C57BL/10 mice were implanted with the B10.2 tumor and treated with a suboptimal regimen of Flt3L (days 1–14) and/or injected with 41BB (M6) mAb on days 13 and 16. Treatment with either Flt3L or 4-1BB (M6) resulted in tumor rejection in a proportion of mice (40 and 80%, respectively), but combining Flt3L and 4-1BB (M6) treatments resulted in rejection of tumors in 100% of the test mice and at a faster rate than in mice treated with either agent alone (Fig. 8A). In a follow-up experiment, tumors were implanted 10 days before initiating treatment with a more aggressive regimen of Flt3L (20 injections on days 11–30). Injections of 4-1BB (M6) were given on days 24 and 27. Although administration of Flt3L or 4-1BB (M6) alone were effective (resulting in 7 of 10 and 9 of 7 tumor rejections, respectively), the combination resulted in complete tumor rejection in all 10 test mice (Fig. 8B). In both settings, the sizes of tumors in the remaining mice that had been treated with Flt3L or 4-1BB (M6) were smaller than those in the control groups.

**Discussion**

The cell surface receptor 4-1BB (CDw137) is a member of the TNFR superfamily and is expressed on activated NK, CD4, and CD8 T cells. Initial efforts to characterize the biologic function of this molecule demonstrated that ligation by either plate-bound mAb or its cognate ligand (on the surface of transfected cells) provided a potent costimulatory signal that promoted proliferation of T cells in the presence of suboptimal levels of mitogens such as PHA or solid phase CD3 (34–37). Additional observations demonstrated that 4-1BB plays an important role in the generation and regulation of immune responses in vivo have been provided by evaluation of 4-1BB knockout mice in which generation of effective immune responses to viral infections is severely inhibited (44, 45). The possibility that pharmacologic ligation of 4-1BB could affect the generation of immune responses in vivo was initially demonstrated by Shuford et al. (35), who found that treatment of mice with 4-1BB-specific mAb augmented the generation of allospecific immune responses in vivo. Additional studies demonstrated that treatment of tumor-bearing mice also promoted the generation of tumor immunity (38, 39). Data presented here confirm and extend these earlier observations and provide a clearer picture of the cellular interactions and cytokines involved in generation of immune responses mediated by 4-1BB-specific mAb.

Initial studies in which mice were treated with 4-1BB (M6) mAb at various times after tumor implantation demonstrated not only that this mAb was capable of promoting the generation of effective immune responses to the tumor but also that initiation of treatment relatively soon after tumor challenge was not as effective as initiation of treatment after the tumor had become more established (Fig. 1). This observation suggests that the mAb is acting on cells that have already been activated by tumor Ags in vivo. Studies presented by Gramaglia et al. (36) demonstrated peak expression of 4-1BB on OVA-specific transgenic CD8 T cells at 48–72 h after stimulation in vitro. In an in vivo setting, Takahashi et al. (46) observed a relatively rapid and transient stimulation of 4-1BB expression on a polyclonal T cells subsequent to immunization with the superantigen staphylococcal enterotoxin A, with maximal responses occurring at 12 h. In both of these settings, the stimulatory signal(s) were very strong and relatively short-lived. In the
tumor settings used here, in which the precursor frequencies of responding T cells are undoubtedly quite low, we have not been able to detect significant increases in 4-1BB expression on polyclonal CD8 cells. However, the fact that effective tumor-immune responses can be stimulated in populations of CD8 cells contained in mice as early as 3–6 days and as late as 21–24 days after tumor implantation by 4-1BB-specific mAb indicates the continued functional expression of this important TNFR superfamily member most likely as a result of continued antigenic stimulation by a progressively growing tumor.

Further analysis of the cellular interactions demonstrated that while CD8\(^+\) T cells were absolutely required, CD4\(^+\) T cells, NK cells, and NKT cells were not (Fig. 2) and is in sharp contrast to previously published results by others (38, 39). The reason (s) for the difference in results presented here compared with those obtained by others is not immediately apparent. The possibility that the treatment protocols we used for depletion of these subsets of cells were ineffective seems highly unlikely because flow cytometric analysis of peripheral blood from the treated mice demonstrated complete depletion of the relevant subsets of cells (Fig. 2), and similar evaluation of secondary lymphoid tissues resulted in identical results (data not shown). In addition, genetic approaches using mice deficient in CD4 cells (class II\(^{-/-}\) mice) or NK cells (IL-15\(^{-/-}\) mice) showed concordant results (summarized in Table II). The conclusion that the 4-1BB-specific mAb is acting directly on CD8\(^+\) T cells, and independent of CD4 T cell function, is also bolstered by the fact that Ag-specific expansion of OT-1 T cells is substantially augmented by treatment with 4-1BB (M6) mAb (T. de Smedt, unpublished observations). Finally, in a follow-up study, Melero et al. (42) have also reported generation of tumor-immune responses to be CD4 independent.

One of the interesting findings from these studies concerns the cellular and cytokine requirements for maintenance of functional memory immune responses. The data presented here clearly indicate that class II-restricted CD4\(^+\) T cells are not required during the generation of the initial immune response (Figs. 2 and 4A) but do appear to play a role in either the generation or long term maintenance of memory responses (Figs. 3 and 4B). In contrast to expectations based on results of others who have reported a role for IL-15 in the clonal expansion of CD8\(^+\) T cells with a memory phenotype (47), this cytokine does not appear to be required for the maintenance of such cells at a functional level at least for a time period of up to 3 mo (Fig. 5). Interestingly, recent investigations by Schluns et al. (48) and Goldrath et al. (49) indicate that IL-7 is required for the generation and survival of memory CD8 T cells where as IL-15 is required for basal homeostatic proliferation of memory OVA-specific CD8\(^+\) T cells (OT-1 cells). They also showed that although CD8 memory T cells had not proliferated in the absence of IL-15, they were present at higher numbers than in normal mice. They attributed this to less competition among memory cells in IL-15\(^{-/-}\) mice. However they stated that although IL-15 was not required for the generation of memory cells in the short term, (i.e., 3 mo), their results did indicate IL-15 is required for long term (18 mo) survival. Clearly, additional studies to evaluate and enumerate memory CD8 T cells induced following stimulation of an immune response with 4-1BB mAb are warranted.

Studies performed using cytokine and cytokine receptor knockout mice also yielded additional information of interest. Not unexpectedly, mice that were deficient in their ability to produce IFN-\(\gamma\) were unable to develop effective immune responses to tumors even after treatment with 4-1BB (M6) mAb (Fig. 6). Whether this is due to failure of clonal expansion of the tumor-reactive T cells, a failure of such cells to differentiate into functional effector cells or the inability of effector cells to traffic to the tumor site is unknown, although results of studies presented by Nakajima et al. (50) suggest the latter. However, studies evaluating the role of

### Table II. Effect of 4-1BB (M6) mAb treatment in promoting tumor rejection in gene knockout mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>No. of Expts.</th>
<th>Control IgG TBA/total</th>
<th>% Tumor rejection</th>
<th>4-1BB (M6) TBA/total</th>
<th>% Tumor rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/10 wild-type</td>
<td>5</td>
<td>50/50</td>
<td>0</td>
<td>13/60</td>
<td>78</td>
</tr>
<tr>
<td>IL-15(^{-/-})</td>
<td>2</td>
<td>8/8</td>
<td>0</td>
<td>7/14</td>
<td>50</td>
</tr>
<tr>
<td>IFN-(\gamma)(^{-/-})</td>
<td>1</td>
<td>10/10</td>
<td>0</td>
<td>10/10</td>
<td>0</td>
</tr>
<tr>
<td>MHC class II(^{-/-})</td>
<td>2</td>
<td>14/15</td>
<td>6.7</td>
<td>0/17</td>
<td>100</td>
</tr>
</tbody>
</table>

* Mice were challenged with 5 \(\times\) 10\(^5\) tumor cells (as indicated) by s.c. injection. Groups of mice were treated by injection of either 4-1BB (M6) or rat IgG Ab control (100 \(\mu\)g/injection, administered i.p.) on days 5, 8, 11, and 14.

### Table III. 4-1BB (M6) mAb treatment to promotes tumor immunity in CD40\(^{L/-}\) mice but not CD40\(^{/-}\) mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>No. of Expts.</th>
<th>CD154 TBA/total</th>
<th>4-1BB (M6) TBA/total</th>
<th>% Tumor Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/10 wild-type</td>
<td>3</td>
<td>--</td>
<td>24/24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>12/36</td>
<td>67(^a)</td>
</tr>
<tr>
<td>CD40(^{-/-})</td>
<td>2</td>
<td>+</td>
<td>18/20</td>
<td>10(^b)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>--</td>
<td>11/13</td>
<td>15(^b)</td>
</tr>
<tr>
<td>CD40L(^{-/-})</td>
<td>3</td>
<td>--</td>
<td>17/18</td>
<td>6(^c)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>16/16</td>
<td>0(^c)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>17/27</td>
<td>37(^c)</td>
</tr>
</tbody>
</table>

* Mice were challenged with 5 \(\times\) 10\(^5\) tumor cells (as indicated) by s.c. injection. Groups of mice were treated by injection of either 4-1BB (M6) or rat IgG Ab control (100 \(\mu\)g/injection, administered i.p.) on days 5, 8, 11, and 14. Anti-CD154 M158 mAb was administered at 100 \(\mu\)g i.p. daily starting 1 day before tumor implantation and extending to day 14.

\(^a\) Frequency of tumor rejection compared with rat IgG treatment of wild-type control mice is statistically significant \((p < 0.0001)\).

\(^b\) Frequency of tumor rejection compared with rat IgG-treated wild-type control mice is not statistically significant \((p > 0.05)\).
CD40 and CD40L yielded a couple of surprises. First, studies using a CD40L-specific mAb (M158) that blocks binding of this cytokine to its receptor effectively inhibited the generation of tumor immunity mediated by 4-1BB (M6) (Fig. 7A). Similar result were obtained with CD40L-/- mice. Coupled with the fact that CD4 T cells are not required to develop 4-1BB-mediated effect or cell responses, the data indicate that CD40L/CD40 interactions between CD8 T cells and DC are critically required for T cell expansion and function. Surprisingly, tumor-bearing CD40L-/- mice were able to develop immune responses to tumors when treated with 4-1BB (M6) mAb (Fig. 7B, and summarized in Table III). Evaluation of composite results from four separate experiments demonstrated that although the frequency of 4-1BB (M6)-induced tumor rejection in CD40L-/- mice (37%) was somewhat reduced when compared with wild-type mice (67%; p = 0.024), it was clearly greater than with untreated wild-type mice (0%; p 0.0001) or 4-1BB (M6)-treated CD40L-/- mice (6%; p = 0.0307). It was also greater than that observed in anti-CD40L (M158)-treated mice (10%; p = 0.0467). Collectively, these data suggest that there may be a second ligand for CD40 (CD40L?2) the function (perhaps even expression) of which is observed only when the primary ligand (CD154) is not functionally expressed during the development of the immune system. Clearly, acute blockade of function (such as mediated by treatment with CD40L-specific mAb) is not sufficient to promote expression and/or function of the putative alternate ligand for CD40. The functional signal must also be delivered via CD40 because 4-1BB (M6) treatment of CD40L-/- mice did not result in any detectable benefit as judged by either tumor incidence or growth rate (Fig. 7B).

Finally, we have previously shown that treatment of tumor-bearing mice with Flt3L (a cytokine that promotes the generation of large numbers of DCs in vivo) also results in the generation of effective CD8-mediated immune responses, presumably by enhancing the efficiency of Ag presentation to T cells. A clear cooperative effect was observed when the two treatment protocols were combined in tumor-bearing mice. Thus, the data suggest that not only is effective Ag presentation by DCs required to activate CD8+ T cells and promote 4-1BB expression but also that enhancing the Ag presentation capacity of the host may also enhance the proportion of specifically activated T cells upon which 4-1BB agonists can act.

In conclusion, our studies confirm the observation that agonistic mAb to 4-1BB can promote the generation of effective CD8-mediated immune responses to established tumors in vivo (38) and substantially expand our knowledge of the cellular and cytokine interactions involved in and required for this effect. At the cellular level, interactions between DCs and CD8 T cells appear to be necessary and sufficient to promote priming and activation of T cells to a point at which costimulation through 4-1BB is effective in generating tumor immunity. Interestingly, although CD4 T cells are not required for 4-1BB-mediated augmentation of tumor immunity, they do appear to play a role in the maintenance of functional memory immune responses. The data also indicate that a CD40-mediated signal is required for clonal expansion and differentiation of effector cell function and that this interaction (presumably occurring between the primed CD8 T cell and the DC) occurs subsequent to 4-1BB ligation. IFN-γ-mediated signals are also required for the generation of functional effector cells and this, too, appears to be subsequent to 4-1BB ligation.

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References


