Anti-4-1BB Monoclonal Antibody Enhances Rejection of Large Tumor Burden by Promoting Survival but not Clonal Expansion of Tumor-specific CD8+ T Cells

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ABSTRACT

Anti-4-1BB monoclonal antibody (mAb) has been shown to induce antitumor immunity by a CD4/CD8-dependent mechanism, but its direct effect on tumor-specific CD8+ T cells in tumor rejection is unclear. Here we used transgenic CD8+ T cells against the unmutated tumor rejection antigen P1A to analyze whether this mAb can promote CD8+ T-cell function against large tumors in the absence of CD4+ T-helper cells. RAG-2(−/−) mice were challenged with P1A-expressing plasmacytoma J558. Once tumor size reached a diameter of 0.85–1.75 cm, mice were treated with P1A-specific CD8+ CTL (P1CTL) in conjunction with anti-4-1BB mAb or control IgG. All of the mice showed a partial regression of tumor, but mice treated with anti-4-1BB mAb markedly enhanced tumor rejection, delayed tumor progression, and prolonged survival. Correspondingly, we observed a substantial increase in the number of P1CTL in anti-4-1BB mAb-treated mice. Surprisingly, anti-4-1BB mAb did not accelerate division of the tumor-specific CD8+ T cells, and the increase in tumor-specific T-cell number was due to reduced activation-induced cell death. These results indicate that anti-4-1BB mAb can promote CD8+ T-cell-mediated protection against large tumors in the absence of CD4+ T-cell help by promoting P1CTL survival without increasing initial clonal expansion.

INTRODUCTION

4-1BB is an inducible costimulatory molecule expressed on activated T cells (1) and natural killer 1.1 cells (2). 4-1BB ligand is expressed on APCs, such as dendritic cells (3), B cells, and macrophages (4). The costimulatory signal provided by 4-1BB has been shown to play an important role in CD8+ T-cell responses in a variety of systems, including viral infection (5–7), allograft rejection (8, 9), and tumor immunity (2, 10–15). Several studies have also demonstrated the efficacy of using anti-4-1BB mAb as an agonist to bypass or enhance the natural costimulation provided by APC. It has been reported that mice treated with anti-4-1BB mAb show increased survival or enhanced regression of tumor (2, 10, 11). These effects were both CD8+ and CD4+ T-cell dependent, as depletion of either subpopulation eliminated the antitumor effect. Because the CD8+ T-cell response can be enhanced by CD4+ T-helper cells, the direct effect of anti-4-1BB mAb on CD8+ T cells in vivo is less clear. Here we used transgenic CD8+ T cells against the unmutated tumor rejection antigen P1A to more clearly elucidate the effect of anti-4-1BB mAb therapy on the antitumor CTL immune response. By adoptively transferring transgenic CD8+ T cells into tumor-bearing RAG-2(−/−) mice, we are able to show that the enhanced tumor rejection, delayed tumor progression, and prolonged survival seen with anti-4-1BB mAb therapy can be mediated by CD8+ T cells in the absence of CD4+ T cells.

A second issue addressed in this study is the mechanism behind the enhanced antitumor immunity due to anti-4-1BB mAb. Costimulation through 4-1BB has been reputed to enhance proliferation and promote survival of T cells (1, 8, 16–18). Most of these studies have used in vitro stimulation models, and the relative contribution of these two mechanisms has not been clearly established in vivo, especially in tumor models. Here we used a well-defined tumor model to dissect the contribution of proliferation and survival to CD8+ T-cell immunity. Our results demonstrate that anti-4-1BB mAb can enhance T-cell immunity by promoting survival without affecting proliferation of CTL.

MATERIALS AND METHODS

Experimental Animals. Transgenic mice expressing TCRs specific for tumor antigen P1A35–43:Ld complex have been described (19). BALB/c mice with a targeted mutation of the RAG-2 gene were purchased from Taconic (Germantown, NY).

Cell Lines and Tumorigenicity Assay. BALB/c plasmacytoma J558 transfected with Neo vector (J558-Neo) has been described (20). J558-Neo tumor cells (5 × 106) were injected s.c. in the flanks of mice. Tumor size and incidence were determined every 2–4 days by physical examination.

Antibodies. Anti-4-1BB mAb producing hybridoma, 2.A, has been described (15). Anti-4-1BB mAb was purified from supernatant by a Protein G column. Rat IgG was purchased from Rockland (Gilbertsville, PA) and Sigma (St. Louis, MO).

Flow Cytometry. Cell surface expression of 4-1BB, peripheral blood P1CTL numbers, T-cell division kinetics, and cell survival markers were visualized by flow cytometry. Transgenic T cells were visualized by surface expression of CD8 and TCR-α chain Vα. All of the antibodies used for flow cytometry were purchased from BD PharMingen (San Diego, CA).

Adoptive Transfer of Purified Transgenic T Cells. For some experiments, unpurified spleen cells from RAG2(−/−) × P1CTL were adoptively transferred directly after RBC lysis with ammonium chloride (Sigma). For other experiments, pooled spleen and lymph node cells from BALB/c P1CTL-transgenic mice were incubated with a mAb mixture (anti-CD4 mAb GK1.5, anti-Flk mAb 2.4G2, and anti-CD11c mAb N418). Unbound mAb was removed, and cells were incubated with anti-immunoglobulin-coated magnetic beads. Ab coated cells were removed with a magnet. Enriched CD8+ T cells with no detectable CD4+ T cells were adoptively transferred into tumor-bearing mice via i.p. or i.v. injection. For some experiments, enriched CD8+ T cells were labeled with CFSE as described (21) before adoptive transfer.

In Vitro Cell Division. In one type of experiment, CD8+ T cells were purified from spleens of transgenic P1CTL mice using magnetic separation and labeled with CFSE as described above. Labeled T cells were cultured with irradiated J558-Neo cells, harvested at 24-h intervals, and stained with anti-CD8-CyChrome and anti-Vα8 TCR-PE. In another type of experiment, unpurified CFSE-labeled P1CTL spleen cells were cultured with P1A peptide, and harvested and stained as above.

BrdU Incorporation. Tumor-bearing mice were adoptively transferred with transgenic P1CTL and treated with either rat IgG or anti-4-1BB mAb on days 4 and 6 after adoptive transfer. On days 6, 9, and 11, mice were given an i.p. injection of 1 mg of BrdU (Sigma). On day 12, mice were sacrificed, and splenic cells were stained with anti-BrdU Ab using a BD PharMingen kit.

Received 2/18/02; accepted 4/18/02.

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Support by NIH Grants CA58033, CA69091, and CA82355.

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2 The abbreviations used are: APC, antigen-presenting cell; mAb, monoclonal antibody; Ab, antibody; CFSE, carboxyfluorescein diacetate succinimidyl ester; PE, phycoerythrin; TCR, T-cell receptor.
RESULTS

Anti-4-1BB mAb Therapy Increases Resistance to Large Tumor Burden. To test whether anti-4-1BB mAb could enhance rejection of a P1A-expressing tumor by P1CTL, RAG-2 (−/−) mice were challenged with a s.c. inoculation of the plasmacytoma J558. When mean tumor diameter reached 0.85–1.75 cm, mice were adoptively transferred with 4 × 10^6 spleen cells from RAG-2(−/−) × P1CTL transgenic mice. Because of lack of endogenous TCR rearrangement, T cells from these mice are CD8^+ and CD4^-. On days 4, 6, 24, and 31 after adoptive transfer, tumor-bearing mice received either 100 µg anti-4-1BB mAb or control IgG via i.p. injection. As shown in Fig. 1, both control IgG-treated and anti-4-1BB Ab-treated mice that received P1CTL showed an initial regression in tumor size. However, 4 of 5 mice treated with anti-4-1BB mAb completely rejected their tumors, whereas only 1 of 5 control mice rejected tumors. Among those control mice that did not reject tumor, several mice exhibited a rapid regrowth of tumor and were sacrificed when moribund. The enhanced immunity by anti-4-1BB mAb has been reproduced by two other experiments with RAG-2(+/+) and RAG-2(−/−) transgenic P1CTL. Thus, anti-4-1BB mAb enhances tumor rejection by CD8^+ P1CTL and extends the survival of those mice that do not completely reject tumor in a CD4^+ T cell-independent manner.

Anti-4-1BB mAb Therapy Increases Percentage of Tumor-specific T Cells in Peripheral Blood. To observe the effect of anti-4-1BB mAb therapy on adoptively transferred tumor-specific CD8^+ T cells, the peripheral blood of tumor-bearing mice from Fig. 1 was analyzed at various time points after adoptive transfer by flow cytometry (Fig. 2). During the first 3 weeks after adoptive transfer, mean P1CTL percentages within the peripheral blood lymphocyte gate were similar in both anti-4-1BB mAb and control groups at days 13 and 23, although slightly higher in anti-4-1BB mAb-treated mice. At 31 days after adoptive transfer, the mean percentages of P1CTL in the anti-4-1BB mAb group (64.8%) remained stable, whereas the mean percentage of P1CTL in the control group (32.2%) had dropped to less than half of the anti-4-1BB-treated group. By 38 days, 2.2-fold greater mean percentage of P1CTL was found in the anti-4-1BB group than in the control IgG group. This difference was extremely significant (P = 0.001). To determine whether the high percentage of P1CTL could be maintained for an extended period of time, we ceased anti-4-1BB mAb treatments after day 31, and took a final blood sample 42 days after the last Ab treatment and 73 days after adoptive transfer. At this late time point, the large difference in mean percent of P1CTL was maintained (65% with anti-4-1BB versus 33.2% with control IgG) and was extremely significant (P < 0.0001). P1CTL percentage was also increased among splenocytes in anti-4-1BB mAb treated mice, whereas recruitment of tumor-infiltrating lymphocytes was not enhanced by anti-4-1BB mAb (data not shown).

Anti-4-1BB mAb Does Not Cause Clonal Expansion of P1CTL by Increasing Initial Division Rate. Several groups have demonstrated various patterns of 4-1BB cell surface expression depending on the method of T-cell activation (16–18). To establish the cell surface expression kinetics of 4-1BB on our tumor-specific P1CTL, we activated spleen cells from transgenic P1CTL mice with P1A peptide. 4-1BB expression was analyzed by flow cytometry at 24-h intervals for 4 days after stimulation (Fig. 3). We observed no 4-1BB expression by unstimulated T cells or on day 1 after stimulation but did see a marked increase in 4-1BB expression beginning at day 2 that was sustained through day 4.

The significant expression of 4-1BB on days 2 and 3 prompted us to test whether anti-4-1BB mAb could increase in vitro clonal expansion of P1CTL at these time points. When spleen cells were cultured with P1A peptide for 48 h in vitro, most T cells divided two to three times (Fig. 4a). By 72 h, most T cells divided four to five times. However, division rates of P1CTL treated with anti-4-1BB mAb or control IgG were very similar. Additionally, we tested the division rate of P1CTL stimulated in vitro by irradiated J558 tumor cells in the absence of APCs (Fig. 4b). At 48 h, few cells had divided, but by 72 h the proportion of divided cells had increased significantly. Nevertheless, anti-4-1BB mAb did not appreciably enhance the division of P1CTL. Thus, anti-4-1BB mAb did not promote T-cell division regardless of whether P1CTL were stimulated by tumor cells or by APC presenting P1A antigen. A comparison of Figs. 4, a and b, reveals that direct stimulation of P1CTL by tumor cells is less efficient than stimulation by peptide and APC. Even in this suboptimal setting with no APC, anti-4-1BB mAb had no effect on T-cell proliferation. It is worth noting that cross-linked anti-4-1BB can promote T-cell division in vitro (15).

To determine whether our in vitro observations of P1CTL division rate could be extended to in vivo P1CTL, we analyzed the early division rate of P1CTL in tumor-bearing mice (Fig. 5). RAG2(−/−) mice with a large tumor burden of 1.0–1.5 cm were adoptively transferred with 5 × 10^6 CD8^+ CD4^- enriched CFSE-labeled P1CTL spleen cells and treated with anti-4-1BB mAb or control IgG on the same day. Mice from each treatment group were sacrificed at 1-day intervals, and spleen cells were harvested and stained to visualize dividing P1CTL. We observed similar division rates among P1CTL from anti-4-1BB mAb-treated and control mice at time points ranging from 38 to 95 h using i.p. or i.v. modes of adoptive transfer. These data...
suggest that the higher number of P1CTL observed in mice that received anti-4-1BB mAb therapy does not result from an increase in early division rate.

The limit of detecting cell division rate extends to only about seven or eight divisions, and thereafter the mean fluorescence levels are negligible. For in vivo detection of P1CTL, this limit is reached after ~4 days (Fig. 5). Because it is possible that anti-4-1BB mAb does not begin to affect P1CTL division until after 4 days, CFSE labeling of P1CTL may not detect a delayed increase in division rate. To test if anti-4-1BB mAb increased P1CTL proliferation at a later time point after adoptive transfer, we treated tumor-bearing RAG2(−/−) mice that had been given an adoptive transfer of P1CTL with BrdU. BrdU is a nucleotide analogue that is incorporated during DNA synthesis and, therefore, can be used as an indicator of cell proliferation. Mice were given three i.p. injections of BrdU (1 mg/injection) during the 7 days preceding sacrifice. On day 12 after adoptive transfer, mice were sacrificed, and CD8+ P1CTL were analyzed for BrdU incorporation.

We observed that the percentage of CD8+ cells with a high level of BrdU incorporation (as determined by intracellular staining with FITC-labeled anti-BrdU Ab) might be somewhat lower in anti-4-1BB mAb-treated mice than in control mice (Fig. 6), although this difference was not significant ($P = 0.18$). These results are consistent with our CFSE data showing that anti-4-1BB mAb does not appear to increase proliferation of P1CTL in vivo.

**Anti-4-1BB mAb Reduces Activation-induced Cell Death of P1CTL.** If anti-4-1BB mAb does not increase proliferation of P1CTL, then the increased percentages of P1CTL observed in peripheral blood may be because of an effect of anti-4-1BB mAb on cell survival. To test this potential role of anti-4-1BB mAb on P1CTL survival, we adoptively transferred tumor-bearing RAG2(−/−) mice with P1CTL and subsequently treated the mice with either anti-4-1BB mAb or control IgG. At day 12 or 13, mice were sacrificed, and P1CTL from spleens were tested for their binding to Annexin V, which interacts with inner cell membrane molecules that translocate to the outer cell membrane during early stages of apoptosis. In one experiment in which spleen cells were stained on the same day of harvest (Fig. 7a), we observed a 2-fold increase in Annexin V-positive cells among CD8+ cells from control IgG-treated mice compared with anti-4-1BB mAb-treated mice (16.2% versus 8.3%; $P = 0.02$). In another experiment, pooled spleen cells from adoptively transferred tumor-bearing mice were harvested and incubated overnight in culture before staining the following day (Fig. 7b). We observed that the fraction of CD8+ cells staining positive for Annexin V was again greater in control IgG-treated mice (13.3%) compared with anti-4-1BB mAb-treated mice (8.7%). Because the decline of T cells occurred over a long period, the difference in death rate adequately accounts for the difference in the number of cancer-specific T cells. Taken together, these results suggest that anti-4-1BB mAb treatment can enhance accumulation of P1CTL in vivo by decreasing activation-induced cell death.

**DISCUSSION**

A critical issue in the field of antitumor immunity is the coexistence of both established tumor and tumor-specific T cells in the same patient (22, 23). In animal studies, it has been observed that the presence of tumor-specific CTL does not always lead to tumor rejection (19, 24, 25). Thus, a major challenge to successful immunotherapy of established tumors is the relative lack of efficacy of the tumor-specific CTL response once tumor burden has reached a large size. Adoptive transfer of tumor-specific CTL can consistently mediate rejection of concurrent challenge of tumor cells or even small
established tumors (24). However, once tumor burden passes a certain threshold (0.5–0.7 cm), then adoptively transferred CTL may cause some tumor shrinkage but rarely can cause a complete rejection (26).

To overcome this barrier, we and others have attempted to modulate costimulatory pathways as a means to boost the antitumor T-cell response. In this study, we analyzed the mechanisms behind the enhanced tumor immunity observed with anti-4-1BB mAb therapy. In RAG2(−/−) mice with large J558 tumor burden (>0.85 cm), we observed enhanced tumor rejection, delayed regrowth of tumor in mice not completely rejecting tumor, and prolonged survival with adoptive transfer of tumor-specific CD8+ T cells and treatment with anti-4-1BB mAb. This finding has added clinical significance for human immunotherapy, because most human tumors are discovered only after they have reached a large enough size to be detected. Identifying strategies that can be effective against large tumor burdens is, thus, a key goal in improving immunotherapy.

Because the recipient RAG2(−/−) mice used in our adoptive transfer model lack endogenous T and B cells, and the P1CTL donor mice are also RAG2(−/−), the anti-4-1BB mAb-enhanced rejection that we observed is mediated by a CD4+ T cell-independent mechanism. Previous studies have shown conflicting data regarding the requirement for specific T-cell subsets in anti-4-1BB mAb-mediated tumor immunity using wild-type mice. Several reports have shown a requirement for both CD8+ and CD4+ T cells in anti-4-1BB mAb-enhanced tumor immunity (10, 11), in contrast to the data presented here. Other studies have demonstrated a CD4+ T cell-independent mechanism for anti-4-1BB mAb-mediated tumor immunity, when either anti-4-1BB mAb (15) or 4-1BB ligand-transfected tumor cells were used (12–14). However, the mechanism of CD8+ T-cell stimulation by anti-4-1BB mAb has not been studied in the absence of CD4+ T cells. Therefore, it is somewhat unclear if anti-4-1BB mAb can activate CD8+ T cells directly. Our results observing transgenic CD8+ T cells in a RAG2(−/−) mouse model clearly showed that this is likely to be the case.

We have chosen this transgenic model to clearly discern the mechanism of anti-4-1BB mAb-mediated CD8+ T-cell immunity. Our findings presented here are consistent with those seen in nontransgenic models. One of us (L. C.) has observed an increase in tetramer-positive CD8+ T cells from tumor-draining lymph nodes in anti-4-1BB mAb-treated mice after in vitro restimulation (15). Others have shown increased CD8+ T-cell numbers in animal models of graft versus host disease (8) or superantigen challenge (17). Correspondingly, decreased CD8+ T-cell expansion has also been observed in lymphocytic choriomeningitis virus-infected 4-1BB ligand knockout mice.
mice (5, 7). Thus, the mechanism uncovered here should have general implications for other settings.

The published data on antigen-specific CD8\(^+\) T cells involves acute T-cell responses. However, most human cancer involves a chronic interaction between T cells and cancer cells. It is generally accepted that chronic interaction with antigen often leads to malfunction of T cells (27), which has indeed been documented in the cancer patient (22). Thus, a major challenge in tumor immunotherapy is to enhance function of cancer-specific T cells in a chronic setting. Here we showed that treatment with anti-4-1BB mAb had a long-lasting effect on the survival of cancer-specific T cells concomitant with a better immunity.

An interesting question is whether 4-1BB ligation maintains cell proliferation, enhances cell survival, or both. Most evidence for 4-1BB-mediated enhancement of proliferation has come from \textit{in vitro} experiments, using thymidine incorporation in allogeneic mixed lymphocyte reactions or with simultaneous anti-CD3 mAb administration (1, 3, 8, 9, 16, 18). Whereas these studies do give information about the conditions required for 4-1BB-mediated proliferation, they may not completely reflect what occurs to T cells \textit{in vivo}. Sun et al. (28) showed a slight increase in CD4\(^+\) T-cell division rate at 55 h with anti-4-1BB mAb treatment after adoptive transfer of CFSE-labeled transgenic T cells into wild-type BALB/c recipients immunized with specific antigen. Whereas several groups have shown an overall increase in CD8\(^+\) T cells \textit{in vivo} with anti-4-1BB mAb treatment (8, 15, 17), these increases can be attributed to either increased proliferation or to reduced cell death. None of the publications on the subject have clearly distinguished the two. Using adoptive transfer of CFSE-labeled P1CTL and \textit{in vivo} BrdU incorporation, we observed that anti-4-1BB mAb does not increase P1CTL proliferation \textit{in tumor-bearing mice}. Division rates of CFSE-labeled P1CTL from anti-4-1BB mAb-treated and control mice were very similar, and BrdU incorporation was actually somewhat higher in control mice. These surprising findings led us to examine tumor-specific T cells for indications that anti-4-1BB mAb treatment may reduce T-cell death, which has been demonstrated \textit{in vitro} by Hurtado et al. (16). \textit{In vivo}, Takahashi et al. (17) have shown that normal decline of staphylococ-
cal enterotoxin A-stimulated CD8⁺ T cells can be prevented by anti-4-1BB mAb. Although the authors have suggested that 4-1BB ligation enhanced CD8⁺ T-cell survival, the effect anti-4-1BB on T-cell proliferation was not studied. Here we showed that anti-4-1BB mAb did not increase T-cell proliferation, and yet the mAb decreased Annexin V staining on P1CTL harvested from tumor-bearing mice. Thus, anti-4-1BB mAb therapy can increase the number of cancer-specific T cells by reducing cell death without increasing cell division. However, our results do not rule out that anti-4-1BB mAb can enhance T-cell proliferation in other settings. The lack of effect on T-cell proliferation could be because of absence of CD4⁺ T-cell help.

Regardless of the immunological basis for the split effect of the mAb, our data provide clear-cut evidence that anti-4-1BB mAb can provide a “bona fide” survival signal, as has been proposed before (17).

It has become increasingly clear that cancer-specific T lymphocytes are expanded in patients with a large tumor burden. However, with one notable exception (29), the existence of these T cells has not been demonstrated to promote immunity to cancer (22, 23). Our results clearly demonstrate that anti-4-1BB mAb can increase the efficacy of T cells specific for tumor antigen, even when it has no effect on T-cell proliferation. The long-lasting effect of anti-4-1BB mAb suggests the potential therapeutic value of this approach.

ACKNOWLEDGMENTS

We thank Drs. Peishuang Du and Lisa Yin for animal typing, and Jing Wen for Ab purification.

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