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Administration of Agonistic Anti-4-1BB Monoclonal Antibody Leads to the Amelioration of Experimental Autoimmune Encephalomyelitis

Yonglian Sun,* Xiaoqi Lin,† Helen M. Chen,* Qiang Wu,* Sumit K. Subudhi,** Lieping Chen,§ Yang-Xin Fu**

4-1BB, a member of the TNFR superfamily, is a costimulatory receptor primarily expressed on activated T cells. It has been shown that the administration of agonistic anti-4-1BB Abs enhances tumor immunity and allogeneic immune responses. Paradoxically, we found that the administration of an agonistic anti-4-1BB mAb (2A) dramatically reduced the incidence and severity of experimental autoimmune encephalomyelitis (EAE). Adoptive transfer of T cells from such treated mice failed to induce EAE, whereas anti-4-1BB treatment following adoptive transfer of encephalitogenic T cells did not prevent EAE pathogenesis. These results suggest that anti-4-1BB treatment during the induction phase inhibits autoreactive T cell immune responses rather than preventing T cell trafficking into the CNS. This was substantiated by the observations that draining lymph node cells from anti-4-1BB-treated mice failed to respond to Ag stimulation in vitro. In addition, we found that such treatment initially promotes the activation and proliferation of Ag-specific CD4⁺ T cells but subsequently increases their probability of undergoing activation-induced cell death, thereby inhibiting effector T cell responses. More importantly, 2A treatment also inhibits the relapse of EAE in a clinically relevant murine model of multiple sclerosis. This study indicates that the agonistic Ab against 4-1BB can potentially be used as a novel immunotherapeutic agent for treating autoimmune diseases. The Journal of Immunology, 2002, 168: 1457–1465.

Efficient activation and differentiation of T cells requires two signals: a primary signal initiated by the engagement of a TCR with a MHC/peptide complex and a secondary signal mediated through one of several accessory molecules expressed on the surface of the T cell. CD28 is a well-characterized costimulatory molecule that is constitutively expressed on the surface of T cells, and it provides the primary costimulatory signal for T cell activation through interaction with its ligands, B7-1 and B7-2, expressed on APCs (1). However, studies of immune responses in CD28-deficient mice showed that CD28-independent immune responses exist (2–4). An example of a CD28-independent response involves 4-1BB (CDw137), a member of the TNFR superfamily, which is primarily expressed on activated T cells (5) and NK cells (6). Its natural ligand, the 4-1BB ligand (4-1BBL), is a member of TNF superfamily, and it has been detected on activated B and T cells, macrophages, dendritic cells, mouse lymphomas, and human carcinoma lines of epithelial origin (7–11).

In vitro studies have shown that 4-1BB signaling augments T cell proliferation and cytokine production through both CD28-dependent and -independent mechanisms (4, 5, 7, 12–14). Furthermore, agonistic anti-4-1BB mAbs preferentially stimulate proliferation of and IFN-γ production by CD8⁺ T cells as compared with CD4⁺ T cells (15), suggesting that 4-1BB is a costimulatory molecule primarily for CD8⁺ T cells. In vivo experiments using agonistic 4-1BB mAbs or 4-1BBL-transfected tumor cells have shown that signaling through 4-1BB can induce the preferential expansion of CD8⁺ CTls that recognize and reject tumors and allograft transplants (15–17). Agonistic anti-4-1BB mAb also prevents superantigen-induced T cell death, especially of CD8⁺ T cells (18). In addition, the study of immune responses in 4-1BBL-deficient mice showed that CD8⁺ T cell responses to virus infections were reduced (19, 20). It appears that activation via 4-1BB is required for optimal CD8⁺ T cell-mediated immune responses in vivo.

The function of 4-1BB in CD4⁺ T cell-mediated immunity has been explored, with most studies suggesting that it plays a costimulatory role (10, 12, 21–23). A previous study showed that 4-1BB could provide a costimulatory signal for T cell activation on APCs lacking B7 molecules by using a MHC class II-restricted autoreactive T cell hybridoma and purified CD4⁺ T cells (10). Another study used purified CD4⁺ T cells responding to allogeneic 4-1BB-expressing stimulator cells to demonstrate that 4-1BB ligation could augment CD28-independent cytokine production by T cells (12). Pigeon cytotoxic ε-presenting fibroblast APCs that were transfected with 4-1BBL were used to suggest that 4-1BB engagement on naive CD4⁺ T cells promotes proliferation, cell cycle progression, and IL-2 secretion (21). In addition, both CD4⁺ and CD8⁺ T cell-mediated in vivo alloresponses were...
regulated by 4-1BB/4-1BBL interactions to approximately the same extent (22). 4-1BBL-induced cell division and enhanced CD4+ and CD8+ T cell effector functions with similar efficacy when using purified TCR-transgenic T cells responding to a specific peptide and allogeneic stimulator cells that express 4-1BBL (23).

However, the introduction of the same agonistic anti-4-1BB mAb (1D8 and 3E1) clones that were used to promote tumor rejection and enhancing graft-vs-host disease (GVHD) abrogated T cell-dependent humoral immune responses in vivo, possibly by inducing Th cell anergy (24). To further investigate the role of 4-1BB signaling in CD4+ T cell-mediated immune responses and its effects on autoimmune diseases, we studied its function in the development of experimental autoimmune encephalomyelitis (EAE). EAE is a Th type 1 cell-mediated demyelinating disease of the CNS that is often used as an animal model for human multiple sclerosis. It can be induced in several strains of animals by immunization with various myelin proteins or immunodominant peptide epitopes derived from myelin basic protein, proteolipidoprotein (PLP), or myelin oligodendrocyte glycoprotein (MOG) (25, 26). Th1-type responses are believed to be responsible for EAE pathogenesis, whereas Th2 responses appear to be protective (27, 28). Because the immunodominant autoreactive Ag in this disease has been identified, EAE serves as an excellent model for studying the function of costimulatory molecules in an Ag-specific system.

Interestingly, we found that the administration of an agonistic anti-4-1BB mAb reduced the incidence and severity of EAE by preventing autoreactive T cell immune responses. Taking advantage of DO11.10 TCR-transgenic mice, we were able to monitor Ag-specific CD4+ T cells, and we found that the administration of anti-4-1BB mAb initially promotes CD4+ T cell proliferation but subsequently accelerates their activation-induced cell death (AICD). Therefore, the engagement of 4-1BB by an agonistic Ab may provide a novel approach to effectively depleting autoreactive T cells.

Materials and Methods

Mice

Six- to 10-wk-old C57BL/6, SJL, and BALB/c female mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6 Rag-1−/− male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DO11.10 BALB/c mice were kindly provided by Dr. A. Sperling (University of Chicago, Chicago, IL). All mice were housed in the University of Chicago Animal Care Facilities. In each experiment, age-matched mice were used.

Peptides

MOG35–55 peptide (MEGVYRSPFRVHLYRNGK) and PLP139–151 (HSLGKWLGHPDKF) were synthesized by Alpha Diagnostic International (San Antonio, TX). The peptides were >76% pure as determined by HPLC. Chicken OVA323–339 (ISQAVHAAHAEINAGR) peptide was synthesized by the Divisional Protein-Peptide Core Facility of the University of Chicago. The peptides were >86% pure as determined by HPLC.

Antibodies

Agonistic anti-4-1BB Ab (2A and IgG2a) was generated by immunizing a Lewis rat (Harlan Sprague-Dawley, Indianapolis, IN) with mouse 4-1BBL.5 Hybridomas were produced by fusing rat spleen cells with mouse Sp2/0 myeloma cells. The culture supernatants were screened by ELISA, and the hybridoma secreting the mAb 2A was selected for additional experiments. To prepare 4-1BB Ig fusion protein, the extracellular domain of mouse 4-1BB was amplified from activated spleen cell cDNA using sequence-specific primers and was then fused to the CH2-CH3 domain of mouse IgG2a in an expression plasmid pMIGV, which was trans-

Ten days after immunization, DLN cells were isolated and cultured in 96-well flat-bottom plates at a concentration of 5 × 10^5 cells/well in complete RPMI 1640 medium (Life Technologies) that contained 10% heat-inactivated FCS, 1 mM glutamine, 1% penicillin-streptomycin, 1 mM non-essential amino acids, and 5 × 10^−5 M 2-ME with various concentrations of MOG_{35-55} peptide. Plates were pulsed with [3H]thymidine (Amersham Pharmacia Biotech) at 0.5 μCi/well on day 4 of culture for the final 18 h. Incorporation of thymidine into DNA was measured by liquid scintillation counting, and the mean was calculated from triplicate wells.

Detection of cytokine production by ELISA

Four and 10 days after immunization, DLN cells were isolated and cultured with or without MOG_{35-55} peptide (25 μg/ml) in complete RPMI 1640 medium for 2 or 3 days. Supernatants were collected for the detection of IFN-γ, GM-CSF, IL-10, and IL-4 by ELISA according to the manufacturer’s instructions. All anti-mouse cytokine mAbs and recombinant cytokin standards were purchased from BD PharMingen (San Diego, CA). All cytokine levels were calculated using standard curves with known amounts of recombinant cytokin.

Real-time PCR

Ten days after immunization, DLNs were removed and total RNA was isolated using TRI reagent (Life Technologies) and was then treated with DNase I (Amersham Pharmacia Biotech). Total RNA (5 μg) was reverse transcribed using the First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech). The mRNA of cytokines and GAPDH were detected by real-time RT-PCR using the ABI Prism 7700-sequence detection system (Applied Biosystems, Foster City, CA) as described previously (30). The primer and probe sequences have previously been reported (31). The probes for cytokines or GAPDH were labeled with 6-carboxy-fluorescein or tetrachloro-6-carboxy-fluorescein, respectively, as a reporter dye. All reactions were conducted in triplicate 20-μl reaction volumes containing TaqMan Universal PCR master mixture (Applied Biosystems). PCR amplification conditions were as follows: 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR results were analyzed using the relative standard curve method according to the manufacturer’s instructions. The amount of cytokine mRNA in each sample was corrected for GAPDH. Final results are displayed as the ratio of the amount of cytokine mRNA over that of control group.

5.6-Carboxy-succinimidyl-fluorescein-ester labeling of DO11.10 cells

Before fluorescence labeling, TCR-transgenic lymph node and spleen cells from DO11.10 transgenic mice were isolated, and CD3+ T cells were purified by immunomagnetic column by negative depletion method (StemCell Technologies, Vancouver, British Columbia, Canada). The purified T cells containing >99% of CD4+ KJ1-26+ cells were resuspended in PBS at 2 × 10^6 cells/ml and they were incubated with 10 μM 5.6-carboxy-succinimidyl-fluorescein-ester (Molecular Probes, Eugene, OR) at 37°C for 30 min.

Tracing adoptively transferred cells

To trace Ag-specific T cells in vivo, we took advantage of DO11.10 TCR-transgenic mice (32, 33). CFSE-labeled and unlabeled TCR-transgenic lymph node and spleen cells from DO11.10 transgenic mice were isolated, and the percentage of CD4+ KJ1-26+ cells was measured by flow cytometry analysis. Three to five million CD4+ KJ1-26+ cells were i.v. injected into nonirradiated BALB/c mice. On the same day, recipient mice were s.c. injected with OVA_{223-239} (300 μg) emulsified in CFA in a volume of 0.1 ml distributed among three sites on the hind back and treated with anti-4-1BB or control IgG (150 μg/mouse) i.p. DLN cells from BALB/c recipients of CFSE-labeled DO11.10 cells were isolated 54 h after immunization and analyzed by flow cytometry. DLN cells from BALB/c recipients of nonlabeled cells were isolated 4, 9, and 19 days after immunization and incubated with anti-FeR mAb (2.4.G2; American Type Culture Collection, Manassas, VA) in staining buffer (PBS containing 1% FCS and 0.1% sodium azide) on ice for 10 min to block FeR. FITC-labeled anti-CD4 (BD Pharmingen) and biotinylated KJ1-26 mAb were then incubated with the cells at 4°C for 20 min. After a wash with staining buffer, the cells were stained with PE-labeled streptavidin (Immunootech, Luminy, France) at 4°C for 20 min. Following one wash, cells were collected on a FACScan (BD Biosciences, Mountain View, CA) and were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Detection of apoptosis

Four to 5 days after immunization, DLN cells from immunized BALB/c recipients were isolated and stained with biotin-labeled KJ1-26 mAb followed by PE-labeled streptavidin. Following two washes, the cells were stained with either FITC-labeled annexin V (BD Pharmingen) or 7-ami-noactinomycin D (7-AAD; Sigma-Aldrich) and then analyzed by flow cytometry. For staining with 7-AAD, cells were incubated with FACS buffer containing 20 μg/ml 7-AAD at 4°C for 20 min. The fluorescence of 7-AAD was detected by red channel FL-3 of FACScan. KJ1-26-positive cells were gated to analyze annexin V and 7-AAD staining.

Statistical analysis

Comparison of the mean peak disease severity between two groups of mice was analyzed by the Student’s t test.

Results

Anti-4-1BB mAb treatment inhibits the development of active EAE

To address the role of 4-1BB in the development of EAE, C57BL/6 mice were treated with either agonistic anti-4-1BB mAb (2A) or rat IgG control i.p. on the day of the first s.c. immunization with MOG_{35-55} peptide emulsified in CFA. Interestingly, the results showed that 2A treatment greatly inhibited the development of EAE and reduced the disease incidence and severity (Fig. 1A; Table I). All of the control mice developed moderate (2 of 12; clinical score, 0.5–2) to severe (10 of 12; 83.3%; clinical score, 3–4) EAE. Recovery from the disease was not observed in any of the control mice up to 30 days after onset, and similar results were obtained when the mice were treated with PBS (data not shown).

In contrast, in the 2A-treated group, 5 of 12 mice (41.7%) were entirely asymptomatic, whereas the rest (7 of 12; 58.3%) only displayed moderate symptoms (clinical score, 0.5–2). More importantly, complete recovery was observed in virtually all the mildly symptomatic mice 10–15 days after disease onset. The mean maximal clinical score of 2A-treated mice was significantly lower than that of the control group (0.4 for the 2A-treated group vs 3.1 for the control group; p < 0.001).

The presence/absence and degree of EAE were further evaluated by histological examination of spinal cord sections from both control and 2A-treated mice. Fourteen days postimmunization in control mice, dense lymphocytic infiltrate was observed in the meninges and the white matter and gray matter of the spinal cord (Fig. 1B). In contrast, no or only minimal perivascular lymphocytic infiltrate was observed in the sections obtained from mice treated with 2A (Fig. 1B). These results suggest that 4-1BB engagement during the priming stage of an immune response strongly inhibits the infiltration of autoreactive T cells in the CNS and EAE development.

Anti-4-1BB mAb treatment does not prevent adoptively transferred EAE

The mechanism by which anti-4-1BB mAb administration inhibits EAE development may be due to the inhibition of encephalitogenic T cell responses and/or their migration into the CNS. To dissect this issue, we tested the effect of 2A treatment in an adoptive transfer model of EAE. Activated MOG-specific T cells were obtained by coculture for 4 days of MOG_{35-55} peptide with DLN cells from C57BL/6 mice immunized with MOG_{35-55} peptide emulsified in CFA. EAE was established by transferring these activated MOG-specific T cells by i.v. injection into sublethally irradiated C57BL/6 mice that were then treated i.p. with either 2A or control rat IgG at 200 or 500 μg/mouse. The results showed that the administration of anti-4-1BB mAb at the time of adoptive
transfer did not prevent EAE induced by activated effector autoreactive T cells (Fig. 2). This suggests that anti-4-1BB mAb treatment upon adoptive transfer does not prevent the trafficking of activated autoreactive T cells into the CNS, and that 4-1BB plays a role before the effector phase of EAE pathogenesis.

**Anti-4-1BB mAb treatment inhibits MOG-specific T cell responses**

To confirm that anti-4-1BB mAb administration inhibits encephalitogenic T cell responses, pooled DLN cells and splenocytes from C57BL/6 mice, which were immunized s.c. with MOG<sub>35-55</sub> peptide emulsified in CFA in treated i.p. with control rat IgG or 2A, were adoptively transferred by i.v. injection into syngeneic male RAG-1<sup>-/-</sup> mice. The recipients of control IgG-treated lymphocytes developed severe EAE, and in a representative experiment, all control recipients died from EAE 9–12 days after adoptive transfer. In contrast, none of the mice that received 2A treatment developed clinical symptoms by the time the experiment was terminated (2 mo after transfer; Fig. 3A and data not shown). These results suggest that the administration of agonistic anti-4-1BB mAb inhibits autoreactive T cell responses.

### Table I. Clinical parameters of MOG-induced EAE in mice treated with/without anti-4-1BB mAb

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean Maximal Clinical Score (mean ± SEM)</th>
<th>Mean Day of Onset (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe (3–4)</td>
<td>Mild (0.5–2)</td>
<td>None (0)</td>
</tr>
<tr>
<td>Control IgG</td>
<td>10/12 (83.3%)</td>
<td>2/12 (16.7%)</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td>Anti-4-1BB</td>
<td>0/12 (0%)</td>
<td>7/12 (58.3%)</td>
<td>5/12 (41.7%)</td>
</tr>
</tbody>
</table>

*Mice were immunized with 100 μg of MOG<sub>35-55</sub> peptide emulsified in CFA as described in Fig. 1. The differences in clinical score between control and anti-4-1BB mAb-treated mice are statistically significant as determined by Student’s t test (p < 0.001)."
Prevention of the generation of autoreactive T cells by anti-4-1BB treatment in vivo

To determine whether anti-4-1BB mAb treatment impairs in vivo MOG peptide-specific responses, active EAE was induced in C57BL/6 mice, as shown in Fig. 1. Mice were then restimulated in the footpad with MOG35–55 peptide 6 wk after the first immunization, and 24 h later footpad thickness was measured. Naïve mice were used as negative controls. As shown in Fig. 3B, control IgG-treated mice, in contrast to naïve and 2A-treated mice, responded to the MOG peptide. Therefore, 2A treatment inhibited delayed-type hypersensitivity (DTH) responses to MOG peptide, suggesting that anti-4-1BB mAb treatment inhibits the function of Ag-specific T cells.

To further address this possibility, we tested the ability of DLN cells to proliferate in response to MOG peptide stimulation in vitro. DLN cells were isolated from mice 10 days after s.c. immunization with MOG35–55 peptide and i.p. injection of either control rat IgG or 2A. The cells were restimulated with various doses of MOG35–55 peptide in vitro. As shown in Fig. 4A, DLN cells from control rat IgG-treated mice proliferated in a dose-dependent manner to MOG35–55 peptide stimulation, whereas DLN cells from 2A-treated mice responded poorly. This result further supports our in vivo results that anti-4-1BB mAb treatment inhibits the function of autoreactive MOG-specific T cells.

Th1-type responses are believed to be responsible for EAE pathogenesis, whereas Th2 responses appear to be protective (27, 28). To test whether the inhibition of autoreactive T cell function is due to deviation of Th1 to Th2 cytokine production by anti-4-1BB treatment, we detected cytokine production by DLN cells. IFN-γ is an important Th1 cytokine, and the administration of agonistic anti-4-1BB Ab was reported to enhance the production of IFN-γ. Therefore, we determined the potential effects of in vivo anti-4-1BB mAb treatment on MOG-specific production of IFN-γ. C57BL/6 mice were immunized s.c. with MOG35–55 peptide emulsified in CFA and were treated i.p. with 2A or rat IgG. Ten days later, DLN cells were isolated and cultured in medium alone or medium supplemented with MOG35–55 peptide (25 μg/ml) for 2 days. The supernatants were collected, and IFN-γ production was determined by ELISA. When cultured with MOG35–55 peptide, DLN cells from mice treated with control rat IgG produced high levels of IFN-γ (Fig. 4B). In contrast, there was a significant decrease in IFN-γ production by DLN cells from mice treated with 2A. The levels of IFN-γ production by DLN cells cultured with medium alone were very low (data not shown). To determine whether this differential response was due to in vitro artifacts, total RNA was extracted from DLN cells isolated 10 days after immunization for quantification of IFN-γ mRNA using real-time quantitative RT-PCR. As shown in Fig. 4C, expression of IFN-γ mRNA by DLN cells was significantly reduced in 2A-treated mice. These data demonstrate that 4-1BB engagement results in the reduced production of the Th1 cytokine, IFN-γ. To study whether 2A treatment results in increased production of Th2 cytokines, IL-4 and IL-10 production by DLN cells was also measured by ELISA. We found that similar low levels of IL-4 were produced by DLN cells from mice treated with either control rat IgG or 2A, and IL-10 production was low and variable within the two groups (data not shown). These results suggest that agonistic anti-4-1BB does not preferentially induce Th2 cytokines, but instead inhibits a Th1 response to protect the mice from the development of EAE pathogenesis.

Anti-4-1BB mAb treatment does not inhibit the initial expansion of CD4+ T cells, but promotes AICD

The inhibition of Ag-specific CD4+ T cell responses by anti-4-1BB treatment could be due to induction of either anergy or deletion. To discriminate between these two possibilities, we monitored in vivo Ag-specific responses using TCR-transgenic T cells. T cells from DO11.10 TCR-transgenic mice (32), which express transgenes encoding a TCR specific for chicken OVA peptide

![Graph](image1)

FIGURE 2. Anti-4-1BB treatment does not prevent adoptively transferred EAE. C57BL/6 mice were immunized s.c. with 100 μg of MOG35–55 emulsified in CFA. Ten days postimmunization, splenocytes and DLN cells were pooled, cultured with MOG35–55 for 4 days, and then transferred i.v. into sublethally irradiated C57BL/6 mice. Recipients were treated i.p. with 200 μg of anti-4-1BB or control IgG on the day of transfer (four mice in each group). Data are representative of two experiments and represent the mean clinical score plotted against time.

![Graph](image2)

FIGURE 3. Prevention of the generation of autoreactive T cells by anti-4-1BB treatment in vivo. A. Anti-4-1BB-treated lymphocytes do not transfer EAE to RAG-1−/− mice. C57BL/6 mice were immunized s.c. with 100 μg of MOG35–55 emulsified in CFA twice at a 1-wk interval and were treated with 150 μg of purified anti-4-1BB or control rat IgG i.p. on the day of first immunization. One week after the second immunization, DLN cells and splenocytes were isolated, pooled, and transferred i.v. into RAG-1−/− mice (four mice per group). Data are representative of two experiments and represent the mean clinical score plotted against time. B. Reduced DTH in mice treated with anti-4-1BB. Active EAE was induced in C57BL/6 mice as in Fig. 1 and was then injected in the footpad with 15 μg of MOG in 15 μl of PBS 6 wk after the first immunization. Twenty-four hours later, thickness of the footpad was measured. Three mice were in each group.
329–339 bound to I-A\(^d\) class II MHC molecule (34), were transferred i.v. into BALB/c mice and were detected with mAbs specific for CD4 and the clonotypic TCR (KJ1-26; Ref. 35). To determine whether the anti-4-1BB mAb (2A) we used in this study inhibited CD4\(^+$\)/H11001 T cell immune responses by preventing the activation of CD4\(^+$\) T cells, we transferred partially purified CFSE-labeled DO11.10 TCR-transgenic T cells into wild-type BALB/c recipients. The recipients were immunized s.c. with OVA 323–339 peptide emulsified in CFA and were injected i.p. with 2A or control rat IgG. Fifty-four hours later, the fluorescence of transgenic cells in DLN was analyzed by flow cytometry. The results showed that in 2A-treated mice, 70% of the transgenic cells divided more than five generations compared with 45% doing so in control mice (Fig. 5A). These results suggest that anti-4-1BB mAb treatment does not inhibit, but actually promotes, the proliferation of CD4\(^+$\)/H11001 transgenic T cells shortly after immunization.

We then examined the kinetics of the Ag-specific T cells following immunization and treatment with the Abs. BALB/c recipients of unlabeled DO11.10 lymphocytes were immunized with PBS or OVA\(_{323-339}\) peptide and were injected with 2A or control rat IgG as above. Four, 9, and 19 days postimmunization, DLN cells were isolated and stained for CD4 and KJ1-26 double-positive cells. As shown in Fig. 5, DLNs from both control and 2A-treated mice contained much higher percentages (Fig. 5B) and numbers (Fig. 5C) of CD4\(^+$\)/H11001/KJ1-26 cells 4 days postimmunization with OVA peptide compared with those from unimmunized recipients. This also suggests that anti-4-1BB mAb administration does not inhibit the initial proliferation of transgenic T cells in

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**FIGURE 4.** Lack of MOG-specific T cell responses after treatment with anti-4-1BB. C57BL/6 mice were immunized s.c. with 100 \(\mu\)g of MOG\(_{35-55}\) emulsified in CFA and were treated i.p. with 150 \(\mu\)g of anti-4-1BB mAb or rat IgG. Ten days later, DLN cells from control IgG or anti-4-1BB-treated mice were isolated and cultured with various concentrations of MOG\(_{35-55}\) peptide in complete RPMI 1640 medium for 5 days for proliferation analysis (A) or 2 days for detection of IFN-\(\gamma\) production (B). A, For proliferation, cells were pulsed with \([\text{H}]\)thymidine for the final 18 h. Incorporation of thymidine into DNA was measured by liquid scintillation counting, and the mean was calculated from triplicate wells. These results are representative of four experiments. B, For IFN-\(\gamma\) production in vitro, the supernatants were collected and analyzed by ELISA. C, For IFN-\(\gamma\) production in vivo, total RNA was extracted from DLN cells, and real-time RT-PCR was performed as described in Materials and Methods. The results are representative of three experiments.

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**FIGURE 5.** Anti-4-1BB treatment does not inhibit the expansion of CD4\(^+$\) T cells, but increases the deletion of activated cells. BALB/c recipients of DO11.10 T cells were immunized s.c. with PBS (Naive) or 300 \(\mu\)g of OVA peptide emulsified in CFA and injected i.p. with 150 \(\mu\)g of control IgG (Control) or anti-4-1BB (Anti-4-1BB). A, Recipients of CFSE-labeled DO11.10 T cells were sacrificed 54 h after immunization, and DLN cells were isolated and examined by flow cytometry. Profiles were gated for KJ1-26\(^+$\) cells. B, Mice were sacrificed 4, 9, and 19 days after immunization, and DLN cells were isolated, counted, and stained with anti-CD4 and KJ1-26 mAbs. C, CD4 and KJ1-26 double-positive cell number in DLN from BALB/c recipients of DO11.10 T cells immunized with PBS (Naive) or 300 \(\mu\)g of OVA peptide emulsified in CFA in addition to control IgG (Control) or anti-4-1BB (Anti-4-1BB). D, Mice were sacrificed 4–5 days after immunization, and DLN cells were stained with KJ1-26 and annexin V or 7-AAD. The fluorescence intensity of annexin V and 7-AAD in KJ1-26\(^+$\) cells is shown. These results are representative of three experiments.
response to OVA323-339 peptide immunization in vivo. However, the percentages and numbers of CD4+ KJ1-26+ cells in the DLNs of 2A-treated mice decreased more rapidly on days 9 and 19 than in control mice (Fig. 5, B and C). Furthermore, analysis of CD4 and KJ1-26 double-positive cells in the spleen and mesenteric lymph nodes revealed similar results (data not shown). These results indicate that although anti-4-1BB mAb administration does not inhibit the initial proliferation of transgenic T cells in response to OVA323-339 peptide stimulation, it does facilitate the clearance of activated T cells, and therefore does not appear to induce anergy.

To dissect how anti-4-1BB mAb treatment increases the clearance of activated T cells, BALB/c recipients of DO11.10 T cells were immunized and treated as above. Four to 5 days later, DLN cells were isolated and stained for KJ1-26 and annexin V or CD4 and KJ1-26 double-positive cells in the spleen and mesenteric lymph nodes showed increased apoptosis and live/dead discrimination, respectively. The results showed a significant increase in the apoptosis of activated cells in 2A-treated mice compared with controls (16% vs 6% by staining with annexin V and 19% vs 7% by staining with 7-AAD (Fig. 5D)). Therefore, anti-4-1BB mAb treatment increases AICD of Ag-specific CD4+ T cells, which could serve as a potential mechanism that regulates the effector Ag-specific T cells.

Anti-4-1BB mAb treatment inhibited MOG-specific Th1 cytokine production at 10 days, but not 4 days, postimmunization

Our study in CD4+ TCR-transgenic T cells showed that agonistic anti-4-1BB mAb treatment initially promotes the proliferation and subsequently increases the depletion of activated CD4+ T cells by increasing AICD. To determine whether this also occurs in an EAE model, we examined the function of MOG peptide-specific CD4+ T cells, and 4 and 10 days postimmunization and Ab treatment, by detecting Th1 cytokine production in DLN cells. The results showed that 4 days postimmunization, 2A-treated DLN cells produced higher levels of IFN-γ and GM-CSF than control DLN cells when cultured with MOG peptide in vitro for 3 days (Fig. 6, A and C). However, 10 days postimmunization, 2A-treated DLN cells produced much less IFN-γ and GM-CSF than control DLN cells (Fig. 6, B and D). This demonstrates that agonistic anti-4-1BB mAb treatment initially promotes the activation of MOG-specific T cells and then down-regulates their effector functions, most likely by enhancing AICD of autoreactive CD4+ T cells.

Anti-4-1BB mAb treatment inhibits the development of relapsing-remitting EAE and prevents the relapse of EAE

The relapsing-remitting clinical course is a characteristic feature of multiple sclerosis. Immunization of SJL mice with PLP139-151 peptide in CFA results in the development of chronic relapsing-remitting EAE. To test whether the administration of anti-4-1BB mAb alters the development of the relapsing disease, SJL mice were treated with 2A or control rat IgG once on the day of immunization or once a week for a total of three times after disease onset. As expected, mice from the control group developed the typical relapsing-remitting EAE. However, 2A treatment on the day of immunization significantly reduced the severity of disease (Fig. 7A). When treatment was initiated after disease onset, EAE relapse was inhibited (Fig. 7B). These results further confirmed that anti-4-1BB mAb administration induces the amelioration of EAE in various mouse models, and, more importantly, can prevent disease relapse.

Discussion

4-1BB is thought to be a T cell costimulatory molecule, as it has been shown that administration of agonistic anti-4-1BB mAbs promotes the rejection of allografts and established tumors, and it enhances GVHD (15, 16, 22). In this study, we explored the effects of administering an agonistic Ab against 4-1BB (2A) to mice afflicted with EAE, a CD4+ T cell-mediated autoimmune disease. Unexpectedly, our results demonstrated that 2A administration inhibits the development of EAE, which correlated with decreased infiltration of mononuclear cells into the CNS. We believe this is
due to the dual role of the agonistic anti-4-1BB mAb, which initially promotes T cell activation, and subsequently augments the clearance of CD4⁺ T cells. Therefore, the administration of agonistic anti-4-1BB mAb may provide a new immunotherapeutic approach to treating CD4⁺ T cell-mediated autoreactive diseases.

Anti-4-1BB mAb administration may promote T cell clearance by complement- or FcR-mediated depletion of the activated T cells that express 4-1BB, by blockade of 4-1BB-4-1BBL interactions, and by deliverance of an agonistic signal through the 4-1BB receptor. A high dose of anti-4-1BB Ab (2A) Fab (500 μg/injection) enhanced tumor immunity, suggesting that the Fc fragment that initiates complement activation or that FcR-mediated depletion is not essential for T cell costimulation (L. Chen, unpublished data). In addition, we observed that the same anti-4-1BB Ab prolonged CD8⁺ T cell activation while increasing CD4⁺ T cell apoptosis, which is inconsistent with a nonspecific cytolytic mechanism. Furthermore, we could detect 4-1BB-expressing cells 4 days postimmunization in the DLNs of mice injected with MOG peptide and treated with 2A (data not shown). Therefore, we believe that 2A does not cause complement- or Fc-mediated depletion of T cells expressing 4-1BB.

2A could also inhibit CD4⁺ T cell responses by blocking the signals between 4-1BB and 4-1BB expressed on activated T cells. We found that EAE mice treated with anti-4-1BBL mAb could not inhibit MOG-specific T cell responses (data not shown). This strongly suggests that just blocking 4-1BB-4-1BB interactions may not be sufficient to prevent the activation of MOG-specific T cells, and, therefore, 2A must be functioning through some other mechanism.

Our data support the third possibility that 2A delivers an agonistic signal through 4-1BB, as demonstrated by increased in vivo proliferation of DO11.10 TCR-transgenic T cells (Fig. 5A) and augmented in vivo cytokine production by MOG-specific T cells shortly after immunization (Fig. 6, A and C). Although the agonistic signal through 4-1BB was able to initially activate CD4⁺ T cells, we unexpectedly observed that it subsequently increased their clearance (Fig. 5, B and C). Therefore, we have demonstrated that 4-1BB can costimulate CD4⁺ T cells and then promote their AICD. Our results differ from previous published data that demonstrated that 4-1BB signaling provides a survival signal for activated lymphocytes (18, 23, 36). One possible explanation is that various T cell subsets could differently respond to 4-1BB-mediated costimulation in vivo. It has been shown that the administration of agonistic anti-4-1BB mAb induces CD8-mediated rejection of established tumors and enhances allograft rejection and GVHD (15, 16). It appears that anti-4-1BB-mediated CD8⁺ T cell responses last longer than CD4⁺ T cells do (15). Takahashi et al. (18) demonstrated that 4-1BB ligation is much more effective in preventing the death of superantigen-activated CD8⁺ T cells than CD4⁺ T cells in vivo. Our study clearly shows that treatment with agonistic anti-4-1BB mAb promotes the deletion of activated CD4⁺ T cells. Taken together, these results suggest that 4-1BB engagement may preferentially provide survival signals for CD8⁺ T cells in vivo.

In accordance with the majority of studies, we found that 4-1BB ligation by an agonistic mAb promotes the activation of CD4⁺ T cells by increasing their ability to proliferate and produce cytokines. By using the TCR-transgenic DO11.10 mice, Cannon and colleagues (23) demonstrate that 4-1BBL promotes CD4⁺ T cell survival in vitro. However, in this study we used DO11.10 mice and observed the dual roles of 4-1BB on CD4⁺ T cell-mediated immune responses in vivo. 4-1BB initially provided T cells with costimulatory signals, and then it unexpectedly decreased their longevity via AICD. This discrepancy can be attributed to the complex nature of an in vivo immune response.

Our result is in contrast to the studies by Blazar and colleagues (22), which showed that CD4⁺ T cell-mediated alloresponses were enhanced by agonistic anti-4-1BB mAb. This could be due to differences among various immune responses. During an allogeneic T cell response in which MHC molecules are mismatched across the MHC, a high proportion of T cell repertoires are involved, and the response could be more acute than the Ag-specific autoimmune disease model we used. Therefore, 4-1BB may have several functions that depend on the activation status of the cell, subset of cell involved, and the type of immune response initiated. Further studies are needed to elucidate mechanisms by which anti-4-1BB stimulation promotes the AICD of CD4⁺ T cells in vivo.

In summary, we were able to inhibit autoreactive T cell responses, and therefore prevent EAE pathogenesis, by treating mice with an agonistic anti-4-1BB mAb. The down-regulation of autoreactive T cell responses could be due to regulation of the Th1/Th2 balance, activation of various subsets of regulatory cells, induction of T cell unresponsiveness (anergy), or deletion of activated cells via AICD. In this study, we have demonstrated that 4-1BB does not appear to affect the Th1/Th2 balance or anergize CD4⁺ T cells. Instead, we have clearly shown that the administration of agonistic anti-4-1BB mAb initially increases T cell activation and then promotes the clearance of these activated CD4⁺ T cells, which results in the attenuation of their effector functions. In support of this dual role of a costimulatory molecule, a recently published study showed that the use of agonistic anti-CD40 Ab initially increased the number of tumor-specific CD8⁺ T cells, and then it accelerated their deletion in a tumor model system (37). In addition, another group has reported that agonistic mAbs against CD40 can successfully control chronic autoimmune inflammatory processes (38). These studies and ours suggest that opposing effects can be elicited by strong T cell stimulation in vivo, and the use of agonistic Abs against costimulatory molecules may help us to reveal the complex functions of these molecules. The agonistic Ab against 4-1BB was able to inhibit two different EAE models, including the murine relapsing model, which is clinically relevant, suggesting that it is possible to preferentially delete activated autoreactive T cells. The use of agonistic Abs against costimulatory molecules may provide a novel strategy for treating T cell-mediated autoimmune diseases.

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References


