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*J Immunol* 2011; 187:1120-1128; Prepublished online 29 June 2011;
doi: 10.4049/jimmunol.1002681
http://www.jimmunol.org/content/187/3/1120

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/06/29/jimmunol.1002681.DC1

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4-1BB Triggering Ameliorates Experimental Autoimmune Encephalomyelitis by Modulating the Balance between Th17 and Regulatory T Cells


A
gonistic anti–4-1BB Ab is known to ameliorate experimental autoimmune encephalomyelitis. 4-1BB triggering typically leads to the expansion of CD8+ T cells, which produce abundant IFN-γ, and this in turn results in IDO-dependent suppression of autoimmune responses. However, because neutralization of IFN-γ or depletion of CD8+ T cell only partially abrogates the effect of 4-1BB triggering, we sought to identify an additional mechanism of 4-1BB–triggered suppression of autoimmune responses using IFN-γ- or IFN-γR–deficient mice. 4-1BB triggering inhibited the generation of Th17 cells that is responsible for experimental autoimmune encephalomyelitis induction and progression, and increased Foxp3+CD4+ regulatory T (Treg) cells, particularly among CD4+ T cells. This was not due to a direct effect of 4-1BB signaling on CD4+ T cell differentiation: 4-1BB signaling not only reduced Th17 cells and increased Treg cells in wild-type mice, which could be due to IFN-γ production by the CD8+ T cells, but also did so in IFN-γ–deficient mice, in that case by downregulating IL-6 production. These results show that although secondary suppressive mechanisms evoked by 4-1BB triggering are usually masked by the strong effects of IFN-γ, 4-1BB signaling seems to modulate autoimmune responses by a number of mechanisms, and modulation of the Th17 versus Treg cell balance is one of those mechanisms. The Journal of Immunology, 2011, 187: 1120–1128.

Received for publication August 9, 2010. Accepted for publication May 20, 2011.

This work was supported by National Cancer Center (Korea) Grants NCC-0810720-1 and NCCF-0890301-1, Ministry of Science, Technology and Innovation (MEST)-2006-04212, Korea Health 21 Research and Development Grant A050260, an Innovative Research award from the Arthritis Foundation (to B.S.K.), and Korea Research Foundation Grant KRF-2005-064-E00001.

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4-1BB (CD137) is a potent costimulatory molecule that has both immune-boosting and immune-suppressing effects (1, 2). It is well-established that anti–4-1BB administration can suppress autoimmune diseases, including collagen-induced arthritis (CIA) (3, 4), experimental autoimmune uveoretinitis (EAU) (5, 6), experimental autoimmune encephalomyelitis (EAE) (7), and systemic lupus erythematosus (8, 9).

Several mechanisms are known to be involved in 4-1BB–mediated immune suppression. In models of CIA and EAU (3, 5), agonistic anti–4-1BB induced Ag-dependent clonal expansion of CD11c+CD8+ T cells, and the IFN-γ produced by these cells suppressed the pathogenic CD4+ T cells. Suppression was mediated by the induction of IDO in dendritic cells (DCs). In EAE, spontaneous lupus, and graft versus host disease models (7–10), anti–4-1BB suppressed disease development by causing the activation-induced cell death and anergy of CD4+ T cells. Finally, anti–4-1BB can generate a population of Ag-specific CD8+ T regulatory cells that suppress the Ag-responding CD4+ T cells, and IFN-γ directly stimulates CD8+ T regulatory cells to evoke TGF-β–based suppression of CD4+ T cells (11, 12). These observations indicate that anti–4-1BB treatment can lead to suppression, anergy, and deletion of pathogenic CD4+ T cells, which generate autoimmune inflammation via several different mechanisms. Nevertheless, it is not known what role anti–4-1BB plays in each subset of CD4+ T cells during inflammatory responses.

Th17 cells are currently thought to mediate the inflammation associated with several autoimmune diseases, including EAE and CIA (13–18). However, several observations have undermined the view that the pathogenesis of autoimmune diseases can be straightforwardly explained in terms of the Th1 and Th2 subtypes (18). Thus, recent evidence suggests that Th1 and Th17 cells, but not Th2 cells, play critical roles in the induction of EAE (19, 20). Therefore, it is possible that anti–4-1BB is involved in the regulation of Th17 responses, as well as of Th1 and Th2 responses, during EAE development. It is also unclear whether 4-1BB triggering suppresses EAE by affecting the proliferation and differentiation of CD4+CD25+ regulatory T (Treg) cells, which are the crucial cellular factors protecting against several autoimmune diseases (21–23).

Because IFN-γ is known to inhibit the development of Th17 cells (24–26), in vivo 4-1BB triggering was thought to inhibit Th17 cell development via the production of IFN-γ. However, in a previous study (5), we found that 4-1BB triggering also exerted a moderate suppressive effect on the progress of autoimmune disease in the absence of IFN-γ–mediated signaling. This implied...
the existence of IFN-γ–independent pathways involved in 4-1BB–mediated suppression of autoimmune disease.

In this study, we explored the idea that 4-1BB triggering modulates the development of Th17 cells in both IFN-γ–dependent and –independent ways, and we focused on the nature of the IFN-γ–independent pathway. By comparing the effects of anti-4-1BB mAb in EAE-induced wild-type (WT) and IFN-γ–deficient (GKO) mice, we have characterized a second mechanism responsible for the 4-1BB–mediated suppression of autoimmune responses.

Materials and Methods

Mice and reagents

WT, GKO, and IFN-γr–deficient (GRKO) C57BL/6 (B6) mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All were maintained under specific pathogen-free conditions in the animal facility of the National Cancer Center (Goyang, Korea). Anti-m4-1BB–producing hybridoma cells (3H3) were a gift from Dr. R. Mittler (Emory University, Atlanta, GA). Hybridomas that produce anti-mCD4 (GIK1.5), anti-mCD8 (2.43), and anti-NK1.1 (PK136) were purchased from the American Type Culture Collection. The Abs were produced from ascites and hybridoma culture supernatants and purified using protein G columns (Sigma-Aldrich, St. Louis, MO). Purified rat IgG was obtained from Sigma-Aldrich and served as control Ab. The following mAbs for flow cytometry were purchased from BD Pharmingen: FITC– or PE-Cy5-anti-CD3 (56-6.7), FITC– or PE-Cy5-anti-CD4 (H129.19), PE– or PE-Cy5-anti-CD11c (HL3), PE–anti-IFN-γ (XMG1.2), PE–anti-IL-17 (TC11-18H10), and purified anti-CD19. PE-anti–IL-12p70 (2H7) and FITC–anti-mouse Foxp3 (FJK-16s) from eBioscience were purchased from eBioscience (San Diego, CA). Myelin oligodendrocyte glycoprotein (MOG)35–55 Peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Peptron (Daejeon, Korea). The peptides were 76% pure as determined by HPLC. Recombinant mouse IFN-γ was purchased from R&D Systems (Minneapolis, MN).

EAE induction and Ab treatment

To induce EAE, mice were immunized s.c. at two sites on the flank, with a total of 100 μg of MOG35–55 peptide emulsified in an equal volume of CFA (Life Technologies, Gaithersburg, MD) containing 2 mg/ml Mycobacterium tuberculosis H37 RA (Difco, Detroit, MI) on day 0. The mice also received i.v. injections of 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) on days 0 and 2 and were administered i.p. 200 μg anti-IFN-γ or rat IgG on days 0, 2, 4, and 8. Rat IgG was used as isotype control for the anti–4-1BB mAb. In some experiments, EAE-induced mice were treated with 400 μg anti-CD8 mAb (2.43), anti-CD4 mAb (GK1.5), and anti-NK1.1 mAb (PK136) to deplete CD8+ T, CD4+ T, or NK1.1+ cells, respectively, five times at 5-d intervals from postimmunization (PI) day 0. Mice were scored daily for signs of EAE according to the following clinical scoring system: 0, no clinical signs; 1, tail weakness or paralysis; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis; and 5, moribund or dead animals.

Flow cytometry and calculation of absolute cell numbers

Single-cell suspensions were prepared from draining lymph nodes (DLNs). They were incubated with the Fc blocker 2.4G2 for 10 min at 4°C and stained with specific Abs for surface markers. For intracellular IFN-γ and IL-17 cytokine staining, DLN cells were prepared from MOG-immunized mice on PI day 10 and 16 and stimulated for 6 h with 50 ng/ml PMA and 500 ng/ml ionomycin in medium containing brefeldin A. The cells were first incubated with 2.4G2 for 10 min at 4°C, stained with FITC-conjugated anti-CD4, anti-CD8, and anti-CD11c, fixed and permeabilized with a Cytofix/cytoperm kit (BD Pharmingen), and incubated with PE-conjugated anti-IFN-γ or anti-IL-17. All samples were subsequently analyzed on a FACSCalibur (BD Bioscience). The absolute numbers of each population were calculated by multiplying the percentage measured by flow cytometry by the total number of viable cells (absolute number = percentage × total cells recovered).

Histological analysis

Spinal cords were removed and fixed in 10% formalin, and then embedded in paraffin. Blocks were cut into 6-μm sections and stained with H&E for visualization of inflammatory infiltrates. Histological scores were calculated from histological sections by an investigator blinded to the experimental groups as follows: 0, no infiltration (<50 cells); 1, mild infiltration (50–100 cells); 2, moderate infiltration (100–500 cells); 3, severe infiltration (100–500 cells); and 4, massive infiltration (>500 cells).

Cell isolation

Single-cell suspensions were prepared from the spleens and lymph nodes of mice and preincubated with Fc blocker 2.4G2 for 10 min at 4°C. CD4+ or CD8+ T cells were further isolated by incubating the cells with CD4+ or CD8+ microbeads (Miltenyi Biotec). The CD4+ and CD8+ T cells were >95% pure by flow cytometry. To purify CD11c+ DCs, DLNs and spleens were collected from mice, cut into small pieces, and incubated with collagenase type II (1 mg/ml Sigma-Aldrich) and DNase 1 (15 μg/ml Roche Biotech) at 37°C for 40 min. T cells were depleted twice with CD90+ microbeads, and CD11c+ DCs were further purified from the T cell-depleted cells using CD11c+ microbeads.

RT-PCR

Tissues including DLN, spleen, and spinal cord were taken on day 14 after MOG immunization. Total RNA was extracted using TRizol (Invitrogen Life Technologies) and reverse transcribed into cDNA with SuperScriptII (Invitrogen Life Technologies). Cytokine-specific mRNA was measured by real-time quantitative PCR (Opticon DNA Engine; MJ Research, Waltham, MA). Relative gene expression was determined by the comparative CT method and normalized to the housekeeping gene GAPDH. To simplify presentation, the gene expression data, normalized for GAPDH, are shown as fold increases relative to the levels in the rat IgG group (means ± SD of triplicate experiments).

Production of Th17 cells

CD4+ T cells (5 × 106 cells/ml in 48-well plates) were activated for 72 h with 1 μg/ml anti-CD3 in the presence of 2.5 × 106 CD11c+ cells with various combinations of the following blocking Abs and recombinant mouse cytokines: anti-mouse IFN-γ (5 μg/ml), anti-mouse IL-4 (5 μg/ml), recombinant mouse IL-6 (5 ng/ml), and recombinant human TGF-β1 (1 ng/ml). Depending on the experimental setup, 2.5 μg/ml rat IgG or agonistic anti–4-1BB was added to the cultures. Anti–IFN-γ was omitted when 50 ng/ml IFN-γ was added. The CD4+ T cells were supplemented with fresh medium and reagents on day 3 and cultured on day 4. Cultured cells were stimulated with PMA/ionomycin and 5 μg/ml in the presence of brefeldin A (BD Biosciences). The cells were surface stained with anti-CD4 and stained intracellularly with anti–IL-17 and anti–IFN-γ.

Proliferation assay

Single-cell suspensions from DLNs or spleens were isolated on days 10 and 16 after immunization. They were cultured in 96-well flat-bottom plates at a concentration of 3 × 105 cells/well in complete RPMI 1640 medium, supplemented with various concentrations of MOG35–55 peptide for 72 h, and pulsed with [3H]thymidine (Amersham Biosciences) at 1.0 μCi/well for the final 12 h. Incorporation of thymidine into DNA was measured by liquid scintillation counting, and means of triplicate wells (Wallac) were calculated.

Western blotting of STAT3

CD4+ T cells purified from MOG-immunized mice were lysed in lysis buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na3VO4, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor mixture). Equal amounts of protein were diluted with 4× SDS sample buffer, applied to SDS-PAGE gels, separated, and transferred to nitrocellulose membranes (Millipore). The membranes were blocked, probed with anti-STAT3 (Santa Cruz Biotechnology) or anti-pY-STAT3 (Cell Signaling Technology), followed by appropriate secondary Ab conjugated to HRP, and visualized with the ECL detection system (Amersham Biosciences).

Measurement of cytokines

Culture supernatants were collected from cultures by centrifugation. The cytokines in the supernatants were quantified using a cytometric bead array kit (CBA; BD Biosciences) on a FACSCalibur cytometer equipped with CellQuestPro and CBA software.

Results

IFN-γ–dependent and –independent suppression of EAE by in vivo 4-1BB triggering

To confirm that 4-1BB triggering could suppress autoimmunity in the absence of IFN-γ, we first assessed the severity of EAE in EAE-induced GKO and GRKO B6 mice following treatment with
anti–4-1BB mAb. In agreement with previous findings (7), anti–4-1BB treatment effectively suppressed the development of EAE in WT B6 mice, reducing both the incidence and severity of EAE (Fig. 1A, 1B). IFN-γ played a crucial role in the 4-1BB–mediated suppression of EAE because anti–4-1BB lost most of its therapeutic effect in GKO mice (Fig. 1A, 1B). Nevertheless, it still delayed the onset of EAE by ∼1 wk and decreased the severity of the EAE by 20% (Fig. 1A, 1B). Anti–4-1BB mAb had a similar modest effect in GRKO mice (Fig. 1C, 1D). These effects were not seen in 4-1BB–deficient mice (Supplemental Fig. 1), indicating that they were 4-1BB specific. Histological analysis of spinal cords also yielded consistent findings. Large numbers of infiltrates were found in the spinal cords of rat IgG-treated EAE-induced WT mice but not in mice that received anti–4-1BB mAb (Fig. 1E, 1F). In the absence of IFN-γ, leukocyte infiltration was greater than in the control rat IgG-treated EAE-induced WT mice, and it was reduced a little by exposure to anti–4-1BB mAb (Fig. 1E, 1F). These results confirm that although 4-1BB–mediated suppression of autoimmune disease is primarily mediated by IFN-γ, as previously suggested (3–5), IFN-γ–independent mechanism(s) also contribute to the suppressive effect of 4-1BB.

CD8+ T cell–dependent and -independent suppression of EAE by 4-1BB triggering

Because IFN-γ is mainly produced by CD8+ T cells after treatment with anti–4-1BB mAb (3–5), we next asked whether the IFN-γ–independent suppression was mediated by CD4+ T or CD8+ T cells. Following treatment of EAE-induced WT and GKO mice with anti–4-1BB mAb, the mice were analyzed for CD4+ T versus CD8+ T ratio, IFN-γ production, infiltration of immune cells into the spinal cord, and clinical score, in the absence of CD4+ T, CD8+ T, or NK cells. Consistent with previous findings (3, 5), anti–4-1BB treatment increased the proportion and number of CD8+ T cells and eventually reversed the normal CD4+ T versus CD8+ T ratio even in GKO mice (Fig. 2A, upper panel, 2B). Anti–4-1BB treatment markedly expanded the CD11c+CD8+ T cell population in the absence of IFN-γ (Fig. 2A, lower panel; GKO mice) and led to massive production of IFN-γ from the CD8+ T while barely increasing IFN-γ production in the CD4+ T cells (WT mice; Fig. 2C). Triple staining of CD8+ T cells with CD11c and IFN-γ demonstrated that IFN-γ was primarily produced by the CD11c+CD8+ T cells (Fig. 2D).

When we analyzed the immune cells infiltrated into the spinal cord 14 d after EAE induction, we found that the infiltrated leukocytes were composed of ∼50% of innate immune cells including neutrophils (Gr-1+CD11b+) and monocytes/macrophages (Gr-1−CD11b−), ∼16% of CD4+ T cells, and ∼7% of CD8+ T cells in the case of rat IgG-treated WT mice (Fig. 2E). Treatment with anti–4-1BB mAb increased the proportion of CD8+ T cells to 21% (3-fold increase), whereas the proportion of CD4+ T cells, neutrophils, and monocytes/macrophages decreased. In the spinal cords of EAE-induced GKO mice, there were fewer T cells and more neutrophils and monocytes than in the corresponding rat IgG-treated WT mice. Again, anti–4-1BB mAb reduced the infiltration of CD4+ T cells, neutrophils, and monocytes/macrophages in the GKO mice, and increased CD8+ T cell infiltration.

Because exposure to anti–4-1BB led to massive expansion of IFN-γ/CD8+ T cells (Fig. 2A–C) which are known to contribute to

**FIGURE 1.** IFN-γ–dependent and -independent suppression of EAE by in vivo 4-1BB triggering. A–D, WT, IFN-γ−/− (GKO), or IFN-γR−/− (GRKO) B6 mice (n = 5) were immunized s.c. with 100 μg MOG35–55 peptide emulsified in CFA and injected i.v. with 200 ng pertussis toxin on PI days 0 and 2. They were also injected i.p. with 200 μg agonistic anti–4-1BB (3H3) or rat IgG as isotype control on PI days 0, 2, 4, and 8. Mice were observed daily and scored. Clinical scores (A, C) and percentages of disease incidence (B, D). The data are means ± SD and are representative of three independent experiments. E, Spinal cords were isolated on PI day 16, sectioned at 6-μm thickness, and stained with H&E. Shown is a representative field from the spinal cords. The long arrow dictates mononuclear inflammatory cells in the white matter and short arrow indicates inflammatory foci in the gray matter. Original magnification ×50. F, Histological scores were calculated from a total of 20 sections. The data are means ± SD (**p < 0.001) and are representative of three independent experiments.
the 4-1BB-mediated immunotherapy of autoimmunity (3, 5), we assessed the suppressive effect of anti–4-1BB mAb in the absence of CD8\(^+\) T, CD4\(^+\) T and NK cells.

Depletion of NK cells did not significantly alter the onset and severity of EAE with or without anti–4-1BB mAb treatment, and CD4\(^+\) T cell depletion completely abolished EAE induction (Fig. 2F, 2G). When CD8\(^+\) T cells were depleted, the EAE in both control and anti–4-1BB mAb-treated mice was exacerbated. However, anti–4-1BB mAb still somewhat reduced the severity of EAE despite the absence of CD8\(^+\) T cells (Fig. 2F, 2G) (as it had done in GKO mice). In particular, all the CD8\(^+\) T cell-depleted mice died 15–20 d after EAE induction in the absence of anti–4-1BB mAb treatment, whereas >70% of the mice survived >3 wk with anti–4-1BB mAb treatment, although the severity of their disease was greater than in mice with an intact CD8\(^+\) T cell population (data not shown).

Taken together, these results imply that in vivo 4-1BB triggering suppresses EAE primarily by increasing CD8\(^+\) T cells and IFN-\(\gamma\) production but that a CD8\(^+\) T cell-independent mechanism also contributes to the 4-1BB–mediated suppression of EAE.

**4-1BB triggering suppresses the development of Th17 cells in vivo**

IL-17–producing CD4\(^+\) T (Th17) cells are crucial in the pathogenesis of EAE, and the development of these cells can be inhibited by IFN-\(\gamma\) (24, 25). Because 4-1BB–dependent suppression of EAE was largely eliminated in the absence of IFN-\(\gamma\), it seemed reasonable to expect that 4-1BB triggering suppressed the development of Th17 cells by increasing IFN-\(\gamma\). To test this hypothesis, DLN cells were analyzed for IL-17 production 10 d after EAE induction. Treatment with anti–4-1BB mAb indeed decreased Th17 proportions from 0.8 ± 0.3 to <0.1% in the DLNs of EAE-induced WT mice (Fig. 3A). Th17 cells were 2.5-fold more frequent in the DLNs of EAE-induced GKO mice than in those of WT mice (1.9 ± 0.7% in GKO versus 0.8 ± 0.3% in WT), and their proportion dropped by more than half (to 0.8 ± 0.2%) in response to anti–4-1BB mAb (Fig. 3A). To establish the exact ratio of Th17 cells in WT versus GKO mice, CD4\(^+\) T cells were gated and reanalyzed for IL-17 and IFN-\(\gamma\) production on PI days 10 (Fig. 3B) and 16 (Fig. 3C). There were 2.4 ± 0.3% Th17 cells in rat IgG-treated WT mice, and the proportion decreased to 0.4 ± 0.1% with anti–4-1BB mAb treatment (Fig. 3B). In GKO mice, there were 6.9 ± 0.8% Th17 cells, declining to 4.1 ± 0.6% after anti–4-1BB mAb treatment, a ∼30% decrease. Conversely, IFN-\(\gamma\)-producing CD4\(^+\) T cells were increased 1.7-fold in WT mice by anti–4-1BB mAb (Fig. 3B). Data on absolute numbers of Th17 cells on PI day 10 also indicated that 4-1BB triggering inhibited the development of Th17 cells in the absence of IFN-\(\gamma\) (Fig. 3D). The absolute number of Th17 cells in the anti–4-1BB mAb-treated WT mice was still significantly reduced on PI day 16, although this was not the case in GKO mice (Fig. 3E).

Because Th17 cells are known to cause tissue damage at inflammatory sites, we next tested whether treatment with anti–4-1BB mAb would reduce the infiltration of Th17 cells into the spinal cord. We assessed levels of IL-17 transcripts in spinal cords by real-time RT-PCR on PI day 14 and found that they were decreased by anti–4-1BB mAb in both WT and GKO mice (Fig. 3F).
These results establish that 4-1BB triggering in vivo suppresses the development of Th17 cells via both IFN-γ-dependent and IFN-γ-independent manner.

**Indirect suppression of Th17 cell differentiation by 4-1BB triggering**

The involvement of IFN-γ in inhibition of Th17 cell differentiation has been demonstrated in many studies (24, 25). However, because 4-1BB triggering suppressed the differentiation of Th17 cells through IFN-γ-dependent and –independent mechanism, we wondered whether Th17 cell differentiation would also be suppressed by direct intracellular signaling through 4-1BB. To examine this possibility, we isolated CD4+ T and CD11c+ DCs from WT and GRKO mice and cocultured them in the presence of IL-6, TGF-β, blocking anti–IL-4, and anti–IFN-γ mAb. Th17 cells were generated normally from the CD4+ T cells in vitro and represented 10–12% of the total CD4+ T cells (Fig. 4A, upper panel). Among the GRKO DCs and CD4+ T cells, Th17 cells increased up to 35% in the same conditions. Contrary to our expectation that CD4+ T cells would produce fewer Th17 cells in the presence of 4-1BB signaling, we observed normal numbers of Th17 cells (Fig. 4A).

Because the addition of recombinant mouse IFN-γ to cocultures of WT CD4+ T cells and WT DCs inhibited Th17 cell development effectively (Fig. 4A, lower panel), we concluded that the inhibition of Th17 cell differentiation was not due to an effect of direct intracellular 4-1BB signaling on CD4+ T cells. Moreover, Th17 cell differentiation was also not regulated by direct intracellular signaling through 4-1BB on DCs.

Because direct 4-1BB signaling on CD4+ T cells was not involved in the inhibition of Th17 cell formation, we next considered the idea that the massively expanded population of CD8+ T cells was responsible for the inhibition of Th17 cell formation by 4-1BB triggering. To exclude the involvement of IFN-γ in this inhibition, EAE-induced GKO mice were treated with anti-CD8 mAb as well as anti–4-1BB mAb. Unexpectedly, all the EAE-induced GKO mice died within 2 wk because of depletion of CD8+ T cells, and ~20% of CD8+ T cell-depleted GKO mice were rescued by treatment with anti–4-1BB mAb (data not shown). Because of the increased mortality of CD8+ T cell-depleted GKO mice, we assessed Th17 cell development on PI day 9. Again, we found that treatment with anti–4-1BB mAb in the absence of CD8+ T cells and IFN-γ reduced both the proportion and absolute number of Th17 cells (Fig. 4B, 4C).

These results suggest that the 4-1BB signaling alters the environment in which Th17 cells develop rather than directly regulating CD4+ T cell differentiation. 4-1BB triggering in the absence of IFN-γ increases Treg cells and decreases Th17 cells by reducing IL-6 expression

Because we had found that 4-1BB triggering inhibited Th17 cell differentiation in vivo but not in vitro, we suspected that 4-1BB triggering might only indirectly affect the differentiation of Th17 cells in the absence of IFN-γ. It is well-established that differentiation of naive T cells into Th17 or Treg cells requires upregulation of two different transcription factors, RORγt and Foxp3, and that this is dependent on cytokines TGF-β and IL-6 (27–30). TGF-β alone converts naive T cells into Treg cells that prevent autoimmunity, but TGF-β in the presence of IL-6 induces differentiation of Th17 cells that promote autoimmunity. Therefore, it was possible that 4-1BB triggering regulated the expression of TGF-β and IL-6. To test this possibility, we analyzed transcript levels of TGF-β and IL-6 in the DLNs and spleens of WT and GKO mice. TGF-β levels were comparable in both the DLNs and spleens of the two groups of mice in the absence of anti–4-1BB mAb treatment (Fig. 5A, 5B). However, anti–4-1BB treatment markedly reduced IL-6 expression in the GKO mice but not in the WT mice (Fig. 5A, 5B).

We next prepared DLN cells from EAE-induced mice on PI day 14, stimulated them with MOG35-55 peptide, and measured IL-6 production in culture supernatants. IL-6 production was only elevated in the DLNs of rat IgG-treated GKO mice (Fig. 5C). Because the MOG35-55 peptide is primarily reactive to CD4+ T cells, we concluded that treatment with anti–4-1BB mAb in the absence of IFN-γ reduces IL-6 production by pathogenic CD4+ T cells. To confirm that the CD4+ T cells received signals via IL-6, these T cells were isolated from each group of mice and used to assess the phosphorylation of STAT3 by Western blotting. Consistent with the pattern of IL-6 expression, STAT3 was heavily phosphorylated in the CD4+ T cells of rat IgG-treated GKO mice, but phosphorylation was markedly reduced in the CD4+ T cells of parallel GKO mice with anti–4-1BB mAb (Fig. 5D).
IL17+CD4+ T cells were calculated by multiplying percentages measured
activated for 72 h with 1 mE). Despite this, the ratio of Foxp3 +
tcells. The proportion of Treg cells among the total lymphocytes in
percentage of Treg cells among the CD4+ T cells, and the absolute
Th17 polarization, CD4+ T (5  
for CD11c+ cells and various combinations of Abs and recombinant mouse
tvitrate (MACS; Miltenyi Biotec) after collagenase digestion of tissues. For
by measuring Foxp3 transcripts by real-time RT-PCR. Consistent
in the presence of anti-CD3 mAb and measured cytokine levels in
expression of Th17 cells in the GKO mice. At a molecular level, 4-1BB triggering by downregulating IL-6 seemed to preferentially induce the differentiation of Treg cells rather than Th17 cells in the GKO mice. In contrast, because of the massive production of IFN-γ following 4-1BB triggering (5), the subtle 4-1BB–mediated effects responsible for the enhanced differentiation of Treg cells and the suppression of Th17 cell formation are concealed in WT mice.

4-1BB signaling regulates inflammatory cytokine expression by
CD4+ T cells differently in vitro and in vivo

Because IL-6 was downregulated by 4-1BB triggering in GKO mice in vivo (Fig. 5A, 5B), we wondered whether 4-1BB triggering directly inhibited IL-6 production by CD4+ T cells in the GKO mice. We therefore isolated fresh naive CD4+ T cells from WT and GKO mice, stimulated them for 3 d with anti–4-1BB or rat IgG in the presence of anti-CD3 mAb and measured cytokine levels in the culture supernatants. 4-1BB stimulation enhanced the expression of IL-5, IL-6, IL-13, and IFN-γ in WT CD4+ T cells (Fig. 6A), and the same treatment also increased IL-4, IL-5, IL-13, and IFN-γ but not IL-6 in the GKO CD4+ T cells (Fig. 6B). Expression of all these cytokines by 4-1BB–/–CD4+ T cells was very poor (Fig. 6C). Therefore, we concluded that 4-1BB triggering indirectly downregulates IL-6 production from CD4+ T cells in GKO mice in vivo.

Because we found that 4-1BB stimulation differentially regulated IL-6 expression from CD4+ T cells in GKO mice in vivo and in vitro, we wondered whether other Th2-prone cytokines such as IL-4, IL-5, and IL-13 would be also differentially regulated in vitro and in vivo. Therefore, CD4+ T cells were isolated from WT and GKO mice 16 d after EAE induction and stimulated with various dose of MOG35–55 peptide for 3 d. Treatment of the GKO mice with anti–4-1BB mAb only led to increased expression of IL-5 from their CD4+ T cells not of other Th2-prone cytokines such as IL-4 and IL-13 (Fig. 6D).

Because growth of CD4+ T cells was strongly inhibited by treating WT mice with anti–4-1BB mAb in vivo (Supplemental Fig. 3), we could not conclude whether treatment of anti–4-1BB mAb in vivo modulated the expression of Th2-prone cytokines from CD4+ T cells. However, because CD4+ T cells from the GKO mice still actively proliferated (Supplemental Fig. 3), the preferential expression of IL-5, but not IL-4 and IL-13, in the ex vivo recall response indicated that 4-1BB signaling differentially regulated the expression of Th2-prone cytokines in vitro and in vivo.

Discussion
We have identified an additional mechanism by which 4-1BB triggering suppresses EAE inhibition of Th17 cell production and stimulation of Treg cell production. Evidently, 4-1BB triggering in vivo inhibits the development of EAE in a number of different ways: IFN-γ–dependent induction of IDO in DCs (3, 5, 31), induction of the activation-induced cell death and anergy of CD4+ T cells (7–10), induction of TGF-β on CD8+ T cells (11, 12), and modulation of the balance between Th17 and Treg cell differentiation.

The action of IFN-γ in inhibiting autoimmune diseases has been demonstrated in several experimental models (3, 5, 24–26).
Thus, disease severity is exacerbated in GKO mice or mice treated with blocking anti–IFN-γ mAb due to increased Th17 responses, whereas treatment with recombinant IFN-γ reverses the diseases by reducing Th17 responses. Consistent with a previous report of anti–4-1BB mAb-mediated immunotherapy in a rheumatoid arthritis model (3), CD11c+CD8+ T cells were numbers greatly increased in WT and GKO mice receiving anti–4-1BB mAb, and these cells were the major source of IFN-γ in anti–4-1BB mAb-treated mice (Fig. 2D). Because of the crucial role of IFN-γ in suppressing Th17 cell differentiation (24–26), the IFN-γ–producing CD11c+CD8+ T cells will be the key inhibitors of Th17 cell differentiation. In addition, IFN-γ produced by these cells contributed to the 4-1BB–mediated suppression of pathogenic CD4+ T cells by inducing IDO in DCs and macrophages as previously reported in rheumatoid arthritis and uveoretinitis models (3, 5). 4-1BB triggering again induced IDO expression in DCs and macrophages.

FIGURE 5. 4-1BB triggering inhibits the creation of favorable conditions for Th17 cell development. A and B, Real-time RT-PCR for cytokine-specific mRNA was performed using RNA isolated from the DLNs and spleens of individual MOG-immunized mice on PI day 14 (n = 5). Relative gene expression was determined by the comparative CT method and normalized to GAPDH. Gene expression data, normalized for GAPDH, are shown as fold increases relative to the levels in the rat IgG group. The data are means ± SD (**p < 0.01, ***p < 0.001) and are representative of three independent experiments. C, DLN cells were restimulated with various concentrations of MOG35–55 peptide in complete medium for 3 d ex vivo (n = 5). The production of IL-6 cytokine was determined from the culture supernatants using a CBA kit (BD Biosciences). The plotted data are means ± SD (**p < 0.001). D, CD4+ T cells were purified from MOG-immunized mice on PI day 14 (n = 2) by magnetic bead separation (MACS; Miltenyi Biotec), and expression of total and phosphorylated STAT3 (pSTAT3) was determined by immunoblotting. The results are representative of three independent experiments. E–G, Single-cell suspensions were isolated from DLNs (E–G) and spleen (E) of MOG-immunized mice on PI day 16 (n = 5). Cells were first surface-stained with FITC-anti-CD25 and PE-Cy5-anti-CD4 and then intracellularly stained with PE-anti-Foxp3. All samples were subsequently analyzed on a FACSCalibur. The data are means ± SD (*p < 0.05, **p < 0.01) and are representative of three independent experiments. H, Real-time RT-PCR for Foxp3 gene was performed using RNA isolated from spinal cords (n = 3). Levels of the Foxp3 gene were normalized to GAPDH. The data are means ± SD (*p < 0.001, **p < 0.001) and are representative of three independent experiments.

FIGURE 6. Differential regulation of IL-6 and Th2 cytokine production by 4-1BB signaling. A–C, CD4+ T cells were purified from spleens and lymph nodes of WT (A), GKO (B), or 4-1BB KO (C) B6 mice by magnetic bead separation (MACS; Miltenyi Biotec). Cells were stimulated with 0.5 μg/ml anti-CD3 mAb in the presence of 2.5 μg/ml anti-4-1BB mAb or rat IgG for 72 h. Cytokine levels were measured in the culture supernatants using a CBA kit. The data are means ± SD (***p < 0.001) and are representative of three independent experiments. D, DLN cells were taken from MOG-immunized WT or GKO B6 mice on PI day 16 (n = 3) and restimulated with various concentrations of MOG35–55 peptide in complete medium for 3 d ex vivo. Culture supernatants were collected, and Th1/2 cytokines were determined as before in the culture supernatants. The data are means ± SD and are representative of three independent experiments.
macrophages of EAE-induced mice (Supplemental Fig. 4A, 4B), and the therapeutic effects of anti–4-1BB mAb in WT mice were reversed in IOD-deficient mice (Supplemental Fig. 4C, 4D). These results suggested that 4-1BB triggering in vivo increased IFN-γ production from CD8⁺ T cells, which leads to the amelioration of EAE by suppressing the Th17 cell development and inducing IDO expression in APCs.

Depletion of CD8⁺ T cells may well remove not only the effect of IFN-γ but also that of regulatory CD8⁺ T cells such as CD8⁺ CD122⁺ and CD8⁺CD28⁺ T cells (32–34). Although we have not directly compared the severity of EAE between GKO and CD8⁺ T depletion, we noticed that CD8⁺ T depletion resulted in more severe EAE than GKO (Figs. 1A, 2F). When we analyzed the frequency of CD4⁺ T cells in the presence or absence of CD8⁺ T cells following the induction of EAE, we found that 4-1BB triggering clearly increased the frequency of CD4⁺ T cells in the absence of CD8⁺ T cells contrary to the their effect in WT mice (Supplemental Fig. 5A). 4-1BB triggering decreased Th17 cells and increased Treg cells in the presence or absence of CD8⁺ T cells (Supplemental Fig. 5B, 5C). Although 4-1BB triggering suppressed the IFN-γ-producing CD4⁺ T cells in WT mice that were mainly due to the increase of IFN-γ-producing CD8⁺ T cells, the same treatment massively increased the IFN-γ-producing CD4⁺ T cells in the absence of CD8⁺ T cells (Supplemental Fig. 5D). Because the development of EAE could be mediated by both Th1 and IFN-γ-producing Th1 cells (19, 20), we could guess that the anti–4-1BB was marginally effective to suppress the EAE in the CD8⁺ T cell-depleted mice. Therefore, the therapeutic effects of anti–4-1BB could not be simply explained by the balance of Th1 cells and Treg cells in the absence of CD8⁺ T cells.

In the absence of IFN-γ-mediated signaling, 4-1BB triggering nevertheless reduced Th17 cell differentiation and increased numbers of Treg cells, and this seemed to be due to reduced IL-6 expression (Fig. 5A–C). It has recently reported that, in the presence of TGF-β, IL-6 promotes the development of Th17 cells while inhibiting the development of Treg cells and that blockade of IL-6 suppresses EAE induction by inhibiting the development of MOG-specific Th1 as well as Th17 cells (27–30). Therefore, it appears that IL-6 controls the choice between Th17 and Treg cells in the absence of CD8⁺ T cells.

4-1BB is expressed in various types of immune cells including activated CD8⁺ T, CD4⁺ T, CD4⁺CD25⁺ Treg, DCs, NK/NKT cells, and even B cells (35, 36), and the effects of 4-1BB signaling on each type of immune cells were not the same. Nevertheless, because of the strong suppressive effects of 4-1BB-mediated IFN-γ production, the various fine-tuning effects on non-CD8⁺ T cells resulting from 4-1BB triggering have not previously been apparent. We have shown that 4-1BB triggering decreases the number of Th17 cells and increased that of Treg cells, and that in WT mice this appears to be due to production of IFN-γ, whereas downregulation of IL-6 occurred in the absence of IFN-γ/IFN-γR signaling. It is clear that 4-1BB triggering does not directly suppress IL-6 expression in the CD4⁺ T cells of GKO mice (Fig. 6A, 6B). Because the deficiency of IFN-γ or IFN-γR is entirely artificial, we may have to view the 4-1BB–mediated downregulation of IL-6 as a potential effect of anti–4-1BB. It will be important to clarify the mechanism by which 4-1BB triggering inhibits IL-6 production in vivo in the absence of IFN-γ.

In a separate experiment, we tested whether single injections of anti–4-1BB mAb were effective in treating different stages of EAE. A single injection of anti–4-1BB mAb on PI day 0 or 7, but not on PI day 14, was sufficient to suppress EAE development in B6 mice (Supplemental Fig. 6A). We found that single injections of anti–4-1BB on each of these days increased CD11c⁺CD8⁺ T and Treg cells and reduced Th17 cells (Supplemental Fig. 6B). Because we used an irreversible EAE animal model, we could not be sure whether the single injection on day 14 was insufficient to ameliorate the EAE, or whether it just could not reverse the tissue damage. These results suggest that single injection of anti–4-1BB mAb was sufficient to suppress EAE development and could be effective to treat the relapsed EAE but might not treat the established irreversible EAE.

Taken together, the current study and previous reports demonstrate that 4-1BB triggering ameliorates autoimmunity mainly by the massive production of IFN-γ by CD8⁺ T cells, which induces IDO in APCs (3, 5). However, we have presented evidence above that 4-1BB triggering modulates the balance of Th17 and Treg cells by other routes in a secondary mechanism suppressing EAE in vivo. 4-1BB expression was not restricted to CD8⁺ T cells, and it occurred at different times in different immune cells. Therefore, we suspect that several mechanisms of 4-1BB–mediated immune regulation need to be identified before anti–4-1BB mAb can be safely used in the clinic.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. 4-1BB-mediated amelioration of EAE is a 4-1BB-specific response. WT and 4-1BB−/− C57BL/6 mice were immunized s.c. with 100μg of MOG35-55 peptide emulsified in CFA, and injected i.v. on PI days 0 and 2 with 200ng of pertussis toxin. They were also injected i.p. with 200μg of rat IgG or agonistic anti-4-1BB (3H3) on PI days 0, 2, 4, and 8. Mice were observed daily and scored. Clinical score (A) and percentage of disease incidence (B). Each group contained five mice and all data are means ± SD. Data are representative of at least three experiments with similar results.
Supplementary Figure 2. The frequency and number of Treg cells in EAE-induced mice by in vivo 4-1BB triggering. Single cell suspensions were isolated from DLNs (A-C) and spleen (A) of MOG-immunized mice on PI day 10 (n = 3). Cells were first surface-stained with FITC-anti-CD25 and PE-Cy5-anti-CD4, and then intracellularly stained with PE-anti-Foxp3. All samples were subsequently analyzed on a FACScalibur. Absolute numbers of Foxp3^+CD4^+ T cells were calculated by multiplying percentages measured by flow cytometry by total numbers of viable cells. The plotted data are means ± SD (*, P < 0.05; **, P < 0.01), and results are representative of three independent experiments.
Supplementary Figure 3. 4-1BB triggering suppresses MOG-specific Th cell proliferation. DLN cells were taken from MOG-immunized WT and GKO C57BL/6 mice on PI day 16. They were then re-stimulated with various concentrations of MOG_{35-55} peptide in complete medium for 3 days. The cultures were pulsed with [³H]-thymidine (1 µCi/well) for the final 12 h, harvested onto glass fiber filter mats, and analyzed for [³H]-thymidine incorporation by liquid scintillation counting. The plotted data are means ± SD, and results are representative of three independent experiments. The plotted data are means ± SD, and results are representative of three independent experiments.
Supplementary Figure 4. Indoleamine-2,3-dioxygenase (IDO) is partially involved in anti-4-1BB-mediated amelioration of EAE. Wild or IDO-deficient C57BL/6 mice (n = 5) were immunized s.c. with 100μg of MOG35-55 peptide emulsified in CFA, and were injected i.v. on days 0 and 2 with 200 ng of pertussis toxin. Mice were i.p. treated with 200μg of rat IgG or agonistic anti-4-1BB (3H3) on days 0, 2, 4, and 8 after immunization. (A) RT-PCR for IDO in CD11c+ DCs and CD11b+ monocytes purified from MOG-immunized mice on PI days 10. (B) Frozen sections (5μm thickness) were prepared from DLN at day 10 after immunization and stained with FITC-conjugated anti-mIDO mAb. Sections were viewed and photographed using a laser-scanning confocal fluorescence microscope system (Fluoview FV500, original magnification ×10). (C, D) Clinical scores and percentages of disease incidence.
Supplementary Figure 5. EAE-induced mice were injected i.p. with 200 μg of rat IgG or agonistic anti-4-1BB on PI days 0, 2, 4, and 8, and received 400 μg of anti-CD8 mAb (2.43) on PI days 0 and 5 to deplete CD8+ cells. (A) Draining lymph node (DLN) cells were isolated on PI day 10, and stained with FITC-anti-CD8 and PE-anti-CD4. (B, D) For intracellular cytokine staining, DLN cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hrs. The incubated cells were first surface-stained with FITC-anti-CD4, and then stained intracellularly with PE-anti-IL-17 or -anti-IFN-γ. (C) For Foxp3 staining, DLN cells were first surface-stained with FITC-anti-CD25 and PE-Cy5-anti-CD4, and then intracellularly stained with PE-anti-Foxp3. All samples were subsequently analyzed on a FACScalibur.
Figure 6. The effects of single anti-4-1BB mAb treatment in different state of EAE.

MOG-immunized mice were injected i.p. with 200 µg of rat IgG or agonistic anti-4-1BB on PI days 0, 7, or 14 (n = 5). All mice except for mice that received single injection of anti-4-1BB 14 days after MOG immunization, were scored and analyzed on PI day 14. The remained were scored and analyzed on PI day 21, 7 days after anti-4-1BB treatment. (A) Clinical scores and (B-F) cellular phenotypes. Draining lymph node (DLN) cells were isolated on PI day 14 or 21, and stained with FITC-anti-CD8 and PE-anti-CD4 (B), or FITC-anti-CD8 and PE-anti-CD11c (C). For intracellular staining, DLN cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hrs. The incubated cells were first surface-stained with FITC-anti-CD4, and then stained intracellularly with PE-anti-IL-17 or -anti-IFN-γ (D, F). DLN cells were first surface-stained with FITC-anti-CD25 and PE-Cy5-anti-CD4, and then intracellularly stained with PE-anti-Foxp3 (E). All samples were subsequently analyzed on a FACScalibur. All samples were subsequently analyzed on a FACScalibur. The results are representative of three independent experiments.