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The 4-1BB (a TNFR superfamily member) is an inducible costimulatory molecule that can exert regulatory effects on T cells independently of CD28 stimulation. The in vitro expression of 4-1BB (CD137) is induced following activation of T cells with various stimuli, including anti-TCR mAbs, lectins, and a combination of PMA and ionomycin. To delineate further the physiological role of 4-1BB in immunity, mice deficient in this receptor were generated. These mutant mice develop normally, and were viable and fertile. Humoral responses to vesicular stomatitis virus were comparable with those seen in wild-type mice, whereas the IgG2a and IgG3 isotype responses to keyhole limpet hemocyanin were somewhat reduced in the mutant mice. The 4-1BB-deficient mice demonstrated enhanced T cell proliferation in response to mitogens or anti-CD3 even in the environment of reduced ability to secrete growth-supporting cytokines (IL-2 and IL-4). Although T cells from 4-1BB-deficient mice showed enhanced proliferation, the T cell immune responses of these animals, such as cytokine production and CTL activity, were diminished. In addition, 4-1BB deletion appears to play a role in the regulation of myeloid progenitor cell growth, leading to an increase in these precursor cells in peripheral blood, bone marrow, and spleen. The Journal of Immunology, 2002, 168: 5483–5490.

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4-1BB deletion appears to play a critical role in preventing activation-induced cell death, promoting the rejection of cardiac allografts and skin transplants, enhancing integrin-mediated cell adherence, and increasing the T cell cytolitic potential (reviewed in Refs. 2 and 3), as well as eradicating established tumors (7). Recently, mice deficient in 4-1BB have been generated (8). These gene-deficient mice had normal humoral responses against vesicular stomatitis virus (VSV), decreased CTL responses to influenza virus, and decreased IFN-γ expression under the influence of lymphocytic choriomeningitis virus (8–10). This latter observation supports the finding that 4-1BB-mediated costimulation is critical for CD8+ T cell responses (11). Another interesting feature of the 4-1BB/4-1BB interaction lies in its ability to provide bidirectional signals to interacting APCs. For example, stimulation through 4-1BB by 4-1BB-bearing cells costimulates B cells (12), whereas the triggering of 4-1BB on monocytes by 4-1BBFc plus anti-Fc leads to the secretion of proinflammatory cytokines (13). The molecular mechanisms involved in 4-1BB-mediated signaling are now beginning to be appreciated. Data to date reveal that 4-1BB mediates signals through the TNFR-associated factor/NF-κB-inducing kinase pathway and activates NF-κB (14–16).

To evaluate further the role of 4-1BB in mediating immune responses, mice deficient in 4-1BB expression were generated using homologous recombination in embryonic stem (ES) cells. The 4-1BB-deficient mice developed normally and were fully fertile. These mice were also characterized by dysregulated cell proliferation in response to mitogens, the ability to mount normal in vivo Ag-specific responses, and a capacity to display increased turnover of myeloid progenitors. The 4-1BB-deficient mice showed attenuated graft-vs-host diseases in two marrow graft rejection models (17). The results demonstrate an important role for 4-1BB in regulating some facets of cellular immunity.

Abbreviations used in this paper: 4-1BBL, 4-1BB ligand; ES, embryonic stem; KLH, keyhole limpet hemocyanin; TNP, trimisopropenol; UTR, untranslated region; VSV, vesicular stomatitis virus.
FIGURE 1. A, 4-1BB gene structure. The 4-1BB is structurally related to the members of the nerve growth factor receptor/TNFR superfamily, which are characterized by the presence of three to six patterns of a cysteine-rich motif in their extracellular domains. B, Targeting vector. C, Targeted 4-1BB gene. The targeted 4-1BB gene produced a 6.7-kb SacI fragment, whereas the normal 4-1BB gene produced a 5.5-kb SacI fragment. The ATG initiation codon of 4-1BB was eliminated by Ncol and S1 nuclease digestion. D, Southern blot analysis of 4-1BB/H11001/H11001 mice. The probe was a 1.2-kb SacI/XbaI fragment that lies outside the targeting sequence. The 6.7-kb bands are from targeted copies, and the 5.5-kb bands are from normal copies of the 4-1BB gene. Lanes 1, 3-5, 7, 8, and 11 show the 4-1BB++ allele (5.5 kb); lanes 2, 6, and 9 show the 4-1BB+- allele (6.7/5.5 kb); and lane 10 shows the 4-1BB-- allele (6.7 kb). E, Immuno-fluorescence analysis of 4-1BB expression by splenocytes from 4-1BB++/+-/- littersmates. Filled curves represent immuno-fluorescence staining with isotype-matched control mAb. Open curves represent immuno-fluorescence staining with anti-4-1BB mAb (1AH2). The 4-1BB-- mice were deficient in cell surface 4-1BB expression, as determined by immuno-fluorescence staining. F, T cell proliferation by cross-linking with agonistic anti-4-1BB mAb. Splenic T cells were activated with anti-CD3 in the presence of the indicated plate-immobilized mAbs (500 ng/well) in two independent experiments. The extent of cell proliferation was assessed by incorporating 1 µCi ³H during the last 8 h of a total 48-h cycle. Values are means ± SDs of a representative of three independent experiments.
Materials and Methods

Production of 4-1BB-deficient mice

To clone mouse strain 129/SV genomic DNA containing exons of 4-1BB genes, 129/SV genomic DNA was digested with various restriction enzymes and subjected to Southern blot analysis using the 5' half of 4-1BB cDNA as a probe. BglII restriction enzyme produced a single 7-kb fragment that hybridized to the 4-1BB probe. To clone this fragment, 129/SV genomic DNA (∼100 μg) was first completely digested with BglII and fractionated on 1% agarose gel at 20 V for 16 h. A zone of DNA resolved around the 7-kb fragment was purified by Gene-Clean (Bio 101) and ligated with a BglII-cleaved dephosphorylated pBluescript SK’ vector. The ligation mixture was electroporated into Electro-Max (Life Technologies, Rockville, MD) competent cells. Approximately 2 × 10^8 colonies were screened from the genomic sublibraries with the 4-1BB probe. One colony that was hybridized contained a 7-kb insert. Analysis of the clone revealed that the genomic fragment contained the type 1 5' UTR (exon 1) and exons 2–8 of the 4-1BB gene. The 7-kb BglII fragment was transferred to the BamHI site of the pGem 7 vector.

The 4-1BB gene fragment contained a unique Ncol site at its initiation codon. The gene was cut at Ncol, treated with S1 nuclease, and blunted ended to eliminate the potential for recreation of the ATG codon. A neoymycin gene with a thymidine kinase promoter and polyadenylation signal (Stratagene, La Jolla, CA) was inserted into the Ncol site. The HSV thymidine kinase gene with a polyoma virus enhancer, PYF 411 (a gift from M. Capecchi, University of Utah, Salt Lake City, UT), was inserted at the 3' end of the BglII fragment of the 4-1BB gene. ES cell line E13TGF2a was transfected with the linearized plasmid DNA and selected for G418 and ganciclovir resistance, as previously described (18). Genomic DNA from individual selected clones was digested with SacI and hybridized with a 1.2-kb SacI/XhoI fragment external to the targeting vector to screen for homologous recombination, as previously described (19). A DNA restriction fragment of 5.5 kb corresponded to the wild-type allele, whereas a new 6.7-kb fragment resulted from the targeted allele. Four targeted clones were obtained from the 50 G418 and ganciclovir-resistant ES cell colonies. The targeted ES cells were microinjected into 3- to 5-day-old C57BL/6 blastocysts and transferred to the uterus horn of pseudopregnant C57BL/6 mice. Eight male and seven female chimeras were obtained. We tested for transmission of the mutated allele by mating the chimeras with (C57BL/6 x 129/SV) F1 mice. Male and female offspring were obtained and were subsequently bred with male C57BL/6 and 129/SV mice to transmit the mutated allele. Homozygous 4-1BB-deficient mice (4-1BB−/−) were bred with C57BL/6 or BALB/c mice for at least seven generations to obtain 4-1BB−/− mice on the C57BL/6 or BALB/c background. The diluted samples were subjected to further analysis.

Generation of 4-1BB-expressing cells and activation of 4-1BB−/− cells

The sf-21 cells were infected with wild-type or 4-1BB-expressing recombinant (sf-4-1BB) baculovirus. The cells were washed twice in PBS, fixed in 1% formaldehyde for 15 min at room temperature, then washed three times in PBS. The fixed sf-21 cells (5 × 10^6) were incubated in 96-well plates with 1 × 10^5 splenocytes from 7-wk-old female 4-1BB−/− mice and littermate wild-type (4-1BB+/+) controls. Where indicated, the cultures also received graded concentrations of anti-CD3. Seventy-two hours later, the cells were pulsed with 1 μCi/well [3H]thymidine. Eight hours later, the cultures were harvested and the incorporated radioactivity was measured in a liquid scintillation counter.

Immunization of mice

Eight- to 10-wk-old mice were immunized i.p. with 250 μg keyhole limpet hemocyanin (KLH; Sigma-Aldrich) without adjuvant and boosted 21 days later. Mice were bled before and 5 days after each injection. Isotypes of Abs to KLH were measured by ELISA using HRP-conjugated affinity-purified goat anti-mouse IgM, IgG, IgG2a, IgG2b, IgG3, and IgE (Southern Biotechnology Associates). Mean titers were calculated from three independent experiments. To assess specific Ab production in response to a T-independent Ag, mice were immunized with 50 μg trinitrophenol (TNP)-LPS (Sigma-Aldrich) in saline and bled 7 and 14 days later for determination of anti-TNP Ab level.

Plaque reduction assay

Groups of wild-type and mutant mice were infected with 1 × 10^6 PFU VSV by the i.v. route. The mice were bled 4, 8, 12, and 20 days postinfection. The sera were diluted 40-fold in DMEM/FBS and heat inactivated for 30 min at 56°C. The diluted samples were subjected to further analysis.
serial 2-fold dilution, the resulting samples were mixed with equal volumes of 500 PFU/ml VSV, and the mixtures were incubated for 90 min at 37°C with 5% CO₂. Aliquots of 100 μl serum-virus mix were overlaid on Vero cell cultures, followed by incubation for 60 min at 37°C. At the end of the incubation period, the serum-virus mix was aspirated from the cultures and 0.5% agarose containing DMEM/FBS was added. On the following day, the agarose layer was overlaid with 5% neutral red containing 0.5% agarose-DMEM. The extent of plaque reduction was assessed visually under a light microscope.

VSV-specific CTL responses

The 4-1BB+/+ and 4-1BB−/− littermates on C57BL/6 background were infected with 1 × 10⁶ PFU VSV i.v. Lymph node T cells were harvested 7 days later and tested directly for CTL activity, using the H-2b-restricted, VSV-derived peptide (SDLRGYVYQGLKSG)-pulsed EL-4 cells as target cells (21). ⁵¹Cr-labeled EL-4 target cells (10⁴ cells/well) were added to effectors at ratios from 1:1 to 1:100. After 4 h, a ⁵¹Cr release assay was performed, as previously described (22).

Colony assay

Cells from blood, femur, and spleen of 4-1BB+/+, 4-1BB−/−, and 4-1BB−/− mice were plated at 1 × 10⁴, 5 × 10⁴, and 5 × 10⁵, respectively, in 35-mm tissue culture dishes (Corning, Corning, NY) in 1% methylcellulose culture medium. The culture medium also contained 30% FBS, 1 U/ml Epo (Amgen Biologicals, Thousand Oaks, CA), 10% v/v PWM mouse spleen cell-conditioned medium, a source of multiple growth-stimulating factors, and 0.1 mM hemin, as described elsewhere (23–25). Colonies derived from granulocyte-macrophage, erythroid, and multipotential progenitor cells were scored after 7 days of incubation in a humidified environment in an N₂-O₂-CO₂ incubator (model BNP-210; Taboi ESPEC, South Plainfield, NJ) at 5% CO₂ and lowered (5%) O₂. Absolute numbers

![FIGURE 4. Dysregulated proliferation of splenocytes from 4-1BB−/− mice. Splenocytes from 4-1BB+/+ and 4-1BB−/− littermates were stimulated with various concentrations of anti-CD3 mAb and Con A. [³H]Thymidine incorporation was measured at 48, 72, and 96 h of stimulation. Values are means ± SDs.](http://www.jimmunol.org/)

![FIGURE 5. T cell proliferation in response to 4-1BBL regulatory signals. Splenocytes from 4-1BB+/+ and 4-1BB−/− littermates were stimulated with various concentrations of anti-CD3 mAb (A) and Con A (B) along with sf-21, sf-21 wild-type, and sf-21 4-1BB-expressing cells. T cell proliferation was measured after 72 h of stimulation. Values are means ± SDs.](http://www.jimmunol.org/)
of progenitor cells per organ were calculated based on the number of viable, unseparated nucleated cells per femur or spleen, and the number of viable low density (<1.077 gm/cm³) peripheral blood cells retrieved after density cut separation using Ficoll-Hypaque (Amersham Pharmacia Biotech). The percentage of progenitor cells in S phase of the cell cycle was estimated by the high sp. act. [H]ThdR kill assay (23, 24).

### Results

#### Generation of 4-1BB-deficient mice

The 4-1BB gene (Fig. 1A) was disrupted by inserting a neomycin-resistant gene cassette (Fig. 1B) into the initiation codon of the 4-1BB gene (Fig. 1C). Homologous recombination events in ES cells were screened by Southern blot analysis (Fig. 1D). Of the 50 colonies that were G418 and ganciclovir resistant, four contained the desired mutation. Chimeric mice were obtained by injecting mutant ES cells into C57BL/6 blastocytes. Transmission of the mutated allele was tested for by mating the chimeric mice with (C57BL/6 × DBA/2)F₁ mice. Heterozygous offspring were crossed to generate mice homozygous for the targeted mutation of the 4-1BB gene. To verify inactivation of the 4-1BB molecule, we stained the Con A- or PMA plus ionomycin-stimulated T cell surface with biotin-labeled 1AH2 (anti-4-1BB mAb) and used PE-streptavidin for detection of 4-1BB. The stimulated splenic T cells from homozygous targeted mice did not express 4-1BB, indicating that the 4-1BB locus was disrupted in 4-1BB⁻/− mice. The stimulated T cells from heterozygous (4-1BB⁺/-) mice had approximately one-half the level of 4-1BB expression of normal (4-1BB⁺/+ ) mice (Fig. 1E). Deletion of the 4-1BB gene was further confirmed by stimulating 4-1BB⁺/- and 4-1BB⁻/− cells with a combination of anti-CD3, anti-4-1BB, and anti-CD28 mAbs. The results indicated a significantly reduced proliferation in 4-1BB⁻/− cells, but not in 4-1BB⁺/- cells or anti-CD28-stimulated cells (Fig. 1F).

#### The 4-1BB-deficient mice display normal development

Mice homozygous for the 4-1BB-targeted mutation (4-1BB⁻/− mice) were detected at the expected Mendelian frequency. No abnormalities were observed in the organs of heterozygous or homozygous animals upon gross necropsy or histopathologic examination. The mutant mice were of normal size and weight, and both sexes were fully fertile. To address the issue of 4-1BB deletion on T cell development in primary and secondary lymphoid organs, various lymphoid cell populations were assessed. Flow cytometric analyses of thymocytes, splenocytes, and lymph node cells from 4-wk-old BALB/c wild-type, heterozygous, and homozygous mice revealed no abnormalities in expression of CD4⁺, CD8⁺, B220⁺, or IgM⁺ cells (data not shown). Hence, 4-1BB does not appear to be required for normal T cell maturation or seeding of mature T cells to peripheral lymphoid organs.

#### B cell responses in 4-1BB⁻/− mice

To evaluate Ag-specific B cell responses in 4-1BB⁻/− mice, the mice were immunized with KLH, and serum Ig production in 4-1BB⁻/− and 4-1BB⁺/- littermates was compared. A 2- to 3-fold decrease in IgG2a and IgG3 isotypes was seen in the 4-1BB⁻/− mice, compared with the heterozygous littermates (Fig. 2). Decreased production of IFN-γ in 4-1BB⁻/− mice may explain the decrease in IgG2a because IFN-γ is known to drive IgG2a switching (26). Significant differences between the two genotypes were not seen for any of the other isotypes.

We tested antiviral B cell responses by infecting mutant and wild-type mice i.v. with VSV (Indiana strain, 1 × 10⁶ PFU). Peripheral blood was obtained on days 4, 8, 12, and 20 postimmunization, and sera were analyzed for neutralizing IgM and IgG Abs, as described previously (27). There were no differences in the titers of IgM and IgG in the mutant and wild-type mice (Fig. 3). Thus, despite a slight decrease in the IgG2a and IgG3 responses, overall neutralizing IgG levels were not different, indicating that 4-1BB may not be important in the Ig switching process.

The ability of 4-1BB⁻/− mice to produce specific Ab in response to a T-independent Ag, TNP-LPS, was assessed. Both wild-type and mutant mice exhibited strong IgM anti-TNP titers. Mutant mice produced detectable anti-TNP IgG, but the amounts were not significantly different from those of the wild-type littermates (data not shown).

#### T cell function in 4-1BB⁻/− mice

The 4-1BB was identified on the basis of its ability to enhance T cell activation (2). Therefore, mitogen-induced cytokine production and T cell proliferation were assessed in the mutant and wild-type mice. The results showed that cellular proliferation was significantly increased in the 4-1BB⁻/− mice over a wide range of plate-immobilized anti-CD3 or soluble Con A concentrations and durations of exposure, in contrast to the normal proliferation responses to these mitogens seen in the wild-type mice (Fig. 4).

When we stimulated 4-1BB-deficient T cells with Con A or anti-CD3 in the presence of 4-1BB-expressing sf-21 cells, the proliferative responses were similar to those of the wild-type 4-1BB⁺/+ control (Fig. 5). Because 4-1BBL is also induced on T cells, the 4-1BB/4-1BBL system must have a self-regulating mechanism.

Because of the hyperproliferation seen in the 4-1BB⁻/− T cells, we compared cytokine secretion from T cells obtained from 4-1BB⁻/−, 4-1BB⁺/-, and 4-1BB⁺/+ littermates. Supernatants of
splenocyte cultures collected at 48 h revealed moderately decreased IL-2 and IFN-γ in the mutant mice, whereas IL-4 levels were significantly decreased (Fig. 6). When purified CD4⁺ and CD8⁺ T cells from the mutant mice were stimulated with anti-4-1BB mAb, the levels of IL-2, IL-4, and IFN-γ were also significantly decreased (Fig. 7). These experiments indicate that although 4-1BB/4-1BBL T cells show enhanced proliferation, their capacity for cytokine production is decreased.

To analyze the role of 4-1BB in the development of VSV-specific CTL in vivo, 4-1BB⁺/⁺ and 4-1BB⁻/⁻ littermates on C57BL/6 background were infected with VSV i.v. Lymph node T cells were analyzed for lysis of syngeneic target cells pulsed with H-2b-specific VSV peptide. The results of four independent experiments are shown in Fig. 8. The 4-1BB⁻/⁻ mice showed significantly decreased CTL activity to VSV, compared with wild-type littermates.

Myelopoiesis in 4-1BB⁻/⁻ mice

To further explore a potential role for 4-1BB in the observed dysregulated cellular proliferation, myeloid progenitor cells were examined in bone marrow, spleen, and blood. We found that the absolute numbers of granulocyte-macrophage, erythroid, and multipotential progenitor cells in bone marrow, blood, and spleen were significantly higher in the 4-1BB⁻/⁻ mice, compared with wild-type mice (Fig. 9, A–C). These progenitors were undergoing rapid turnover in the mutant 4-1BB⁻/⁻ mice, as determined by the percentage of cells in the S phase of the cell cycle (Fig. 9D). In the heterozygous 4-1BB⁺/⁻ mice, the absolute numbers of myeloid progenitor cells and their cycling status were intermediate between those of the mutant 4-1BB⁻/⁻ mice and the wild-type 4-1BB⁺/⁺ mice (Fig. 9D). However, there were no apparent differences in nucleated cellularity in bone marrow, blood, and spleen among the three groups of mice. Our finding of dysregulated production of progenitor cells suggests that the 4-1BB/4-1BBL system may also be involved in progenitor cell proliferation, in that the absence of 4-1BB resulted in enhanced cellular proliferation.

FIGURE 7. Cytokine production by purified T cells. CD4⁺ and CD8⁺ T cells were purified as described in Materials and Methods. Forty-eight hours later, supernatants were collected and assayed for IL-2, IL-4, and IFN-γ. Cytokine levels were determined from standard curves. Values are means in picograms per milliliter for IL-2 and IL-4, and in nanograms per milliliter for IFN-γ.

FIGURE 8. CTL responses in wild-type (4-1BB⁺/⁺) and mutant (4-1BB⁻/⁻) mice. C57BL/6 mice (5 wk old) were infected with 1 × 10⁶ PFU VSV i.v., and VSV-specific CTL responses were assessed after 7 days. Lymph node T cells were harvested and diluted with RPMI 1640 medium supplemented with 20 U/ml IL-2 as effector cells. EL-4 cells were labeled with 51Cr with or without VSV-specific peptide for 1 h at 37°C and used as target cells. The target and effector cells were mixed at ratios of 1:100, 1:20, 1:10, and 1:1, and incubated for 4 h at 37°C, after which the 51Cr release assay was performed.
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Another interesting finding concerned the role of 4-1BB in the regulation of precursor cell turnover in primary and secondary lymphoid organs. The study of myeloid progenitor cells indicated growth enhancement in the numbers and cycling status of these cells in 4-1BB−/− mice, suggesting that 4-1BB plays a previously unrecognized role in myelopoiesis. The exact mechanisms associated with enhanced myeloid progenitor cell proliferation in 4-1BB−/− mice are not known, but may reflect the absence of 4-1BB-mediated signals or indirect mechanisms associated with lymphocyte interactions and cytokine productions, as many lymphocyte products are known to be involved in the regulation of blood cell production (25). In this context, we have demonstrated that 4-1BB and 4-1BBL are expressed in a wide variety of tissues and cell types, which has not been recognized previously (30). Detection of surface expression is difficult, but 4-1BB and 4-1BBL can be detected easily by intracellular staining (manuscript in preparation). The mechanism of control for 4-1BB and 4-1BBL surface expression remains to be determined.

References


Discussion

We and others have shown earlier that signaling through the 4-1BB molecule transmits a potent costimulatory signal to T cells in terms of their survival, differentiation, and cytokine expression.(2) It has been shown that blocking 4-1BB/4-1BB interactions either by 4-1BB alkaline phosphatase or by 4-1BB Fc severely curtails T cell proliferation (28). We believed we would find a decrease in T cell proliferation and compromised T cell immunity in 4-1BB-deficient mice. Although we found decreased T cell immune responses, we consistently observed enhanced T cell proliferation with various mitogens in the present studies. The increased proliferation of T cells in 4-1BB-deficient mice upon mitogenic stimulation suggests an as-yet-unidentified role for this costimulatory molecule in T cell growth. When we stimulated 4-1BB-deficient T cells with Con A or anti-CD3 in the presence of 4-1BB-expressing sf-21 cells, the proliferative responses were similar to those of the 4-1BB−/− control. Because 4-1BB is also induced on T cells, the 4-1BB/4-1BB system must have a self-regulating mechanism. The dysregulated proliferation could be the result of the lack of 4-1BB-mediated signals or of indirect mechanisms such as changes in cytokine production. Schwartz et al. (29) observed that cross-linking 4-1BB inhibited T cell proliferation. Therefore, we hypothesized that the 4-1BB provides negative regulatory signals to lymphocytes and progenitor cells. This hypothesis remains to be tested.

In contrast to the dysregulated T cell proliferation in the 4-1BB−/− mice, the T cell immune responses, such as cytokine production and CTL activity, were diminished, suggesting that the proliferative response of 4-1BB−/− T cells is independent of T cell function. When 4-1BB and CD28 are both deficient, the hyperproliferation of T cells seen in the 4-1BB−/− mice disappears (manuscript in preparation). Thus, 4-1BB−/− T cells appear to receive the proliferative signal mainly through CD28, and the absence of 4-1BB-mediated signals leads to T cell hyperproliferation.


