Enhanced CD4 T Cell Responsiveness in the Absence of 4-1BB

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Cells need costimulation for activation, proliferation, and survival, which prevents the induction of tolerance and induces T cell immunity (1). Costimulatory molecules can be divided into two main groups: the Ig superfamily and the TNFR superfamily (2, 3). The 4-1BB (CD137), a member of the TNFR superfamily, was originally found on activated T cells, but has since been identified on other immune cells, including NK cells, monocytes, neutrophils, and dendritic cells (DC) (4–9). The 4-1BB ligand (4-1BBL) is largely expressed on APCs, such as B cells, macrophages, and DC (8, 10).

The interaction of 4-1BB with 4-1BBL was proposed to preferentially costimulate CD8 T cells (11), but a number of studies have demonstrated that it also might play a role in CD4 T cell responses. Engaging 4-1BB on naïve CD4 T cells with 4-1BBL-transfected fibroblast cells promoted proliferation and cell cycle progression and suppressed apoptosis (12). The 4-1BB augmented primary responses of CD4 T cells in the absence of CD28 signaling, and 4-1BB agonistic Ab enhanced CD4 T cell responses in vitro and in vivo (13, 14). Moreover, 4-1BB ligation by single-chain Fv fragments of anti-4-1BB Ab on tumor cells promoted enhanced tumor rejection in a CD4 T cell-dependent manner (15). These results have therefore indicated a positive costimulatory role for 4-1BB on CD4 T cells. In contrast, it has been reported that 4-1BB ligation has either no specific role or even a negative role in regulating CD4 T cells. For example, 4-1BBL−/− mice generated normal CD4 T cell responses after lymphocytic choriomeningitis virus and influenza infection, although CD8 T cell responses were down-regulated in those mice (16, 17). In addition, 4-1BB agonistic Ab treatment unexpectedly led to suppressed pathogenic CD4 T cell responses in several autoimmune disease models, including experimental autoimmune encephalomyelitis, lupus, and collagen-induced arthritis (18–21). Thus, the role of 4-1BB, especially in vivo, appears to be quite complex and may vary depending upon the inflammatory conditions.

The 4-1BB−/− mice develop normally, but were observed to generate decreased CTL responses, although normal Ab responses, following vesicular stomatitis virus infection (22). However, interestingly, splenocytes from 4-1BB−/− mice displayed greater proliferation to mitogens in vitro compared with splenocytes from wild-type mice (22), suggesting again a potential dichotomy between CD4 and CD8 cells. To extend this study, we have specifically addressed the role of 4-1BB in CD4 T cell responses to protein Ag in vivo by first examining T cell priming in a 4-1BB-sufficient environment. Overall, these results reveal a new unappreciated negative regulatory role of 4-1BB when expressed on a T cell. The Journal of Immunology, 2005, 174: 6803–6808.

**Materials and Methods**

**Mice**

The 6- to 8-wk-old male and female C57BL/6 mice were purchased from The Jackson Laboratory. OT-II TCR transgenic mice were used to study OVA123-139 peptide-specific CD4 T cell responses in vivo. The 4-1BB−/− OT-II TCR transgenic mice were generated in house by crossing OT-II mice with 4-1BB−/− mice.

**Purification of CD4 T cells and splenic DC**

CD4 T cells were purified from spleen and lymph nodes (LN) of OT-II or 4-1BB−/− OT-II mice by using a CD4 T cell isolation kit with mid-MACS column (Miltenyi Biotec), according to the manufacturer’s protocol. Splenic DC were purified by MACS. Briefly, spleens of C57BL/6 mice
were pretreated with collagen (Sigma-Aldrich) for 30 min at 37°C. Single cell suspensions of spleen cells were incubated with anti-mouse CD11c microbeads (Miltenyi Biotec) and then positively selected through MACS columns.

**Immunization and adoptive transfer**

Wild-type and 4-1BB/−/− mice were immunized s.c. at the tail base with 50 µg of OVA protein either emulsified in CFA or adsorbed to aluminum (Alum) hydroxide. For adoptive transfer experiments, 1 × 10⁶ Vα2⁺Vβ⁵⁺ CD4 T cells from OT-II or 4-1BB/−/− OT-II mice were injected i.v. into unirradiated wild-type C57BL/6 mice. Two days later, mice were immunized s.c. at the tail base. In some cases, purified CD4 T cells were labeled with 5 µM CFSE (Molecular Probes) for tracking cell division before adoptive transfer.

**ELISPOT assays**

Splenocytes were harvested at day 8 after immunization and processed, as described elsewhere (23). Briefly, multiscreen 96-well plates (Millipore) were coated overnight at 4°C with 4 µg/ml anti-mouse IFN-γ or IL-4 Ab (R4-6A2 or BVD4-1D11; BD Pharmingen). A total of 1 × 10⁵ or 2 × 10⁵ splenocytes were incubated in triplicate wells in the presence of 100 µg/ml OVA protein for 18–24 hr at 37°C. The plates were washed five times and incubated with 0.5 µg/ml biotin-conjugated anti-mouse IFN-γ or IL-4 Abs (XMG1.2 or BVD6-24G2; BD Pharmingen) for 4 hr at room temperature. Plates were subsequently washed five times and treated with streptavidin-alkaline phosphatase, followed by development of spots. Wells were imaged with an AID ELISPOT reader (AID). Spots were counted by an automated system with set parameters for size, intensity, and gradient. Background (mean of wells without OVA protein) levels were subtracted from each well on the plate. Statistical analysis was determined by using Student’s t test.

**In vitro proliferation and ELISAs**

For in vitro recall responses, 3 × 10⁵ splenocytes from immunized mice were stimulated with various doses of OVA protein or OVA₃₂₃–₃₃₉ peptide. Cultures were pulsed with 1 µCi of [³²P]thymidine (ICN Biochemicals) at 60 h and harvested 16 h later. Culture supernatants were harvested 48 h after stimulation to assess production of cytokines (12). To measure OVA-specific Ab responses, sera from immunized mice were collected by tail bleeding at 32 days after immunization. Each Ig isotype was measured, as well as specific Ab responses, sera from immunized mice were collected by tail bleeding at 32 days after immunization. To measure OVA-specific Ab responses, sera from immunized mice were collected by tail bleeding at 32 days after immunization. Each Ig isotype was measured, as described elsewhere (23). Briefly, multiscreen 96-well plates (Millipore) were coated overnight at 4°C with 4 µg/ml anti-mouse IFN-γ or IL-4 Ab (R4-6A2 or BVD4-1D11; BD Pharmingen). A total of 1 × 10⁵ or 2 × 10⁵ splenocytes were incubated in triplicate wells in the presence of 100 µg/ml OVA protein for 18–24 hr at 37°C. The plates were washed five times and incubated with 0.5 µg/ml biotin-conjugated anti-mouse IFN-γ or IL-4 Abs (XMG1.2 or BVD6-24G2; BD Pharmingen) for 4 hr at room temperature. Plates were subsequently washed five times and treated with streptavidin-alkaline phosphatase, followed by development of spots. Wells were imaged with an AID ELISPOT reader (AID). Spots were counted by an automated system with set parameters for size, intensity, and gradient. Background (mean of wells without OVA protein) levels were subtracted from each well on the plate. Statistical analysis was determined by using Student’s t test.

**Flow cytometry**

Ag-activated OT-II TCR transgenic CD4 T cells were stained with anti-CD4 Cy, anti-Vα2 FITC, anti-Vβ⁵ PE, and anti-CD44 allophycocyanin (BD Pharmingen). For 4-1BB detection, cells were stained with anti-4-1BB biotin (Biolegend), followed by streptavidin-PE (Molecular Probes). Analysis was performed on a FACSCalibur with CellQuest software (BD Biosciences), and flow data were analyzed with FlowJo software (Treestar).

**Results**

The 4-1BB/−/− mice generate enhanced CD4 T cell responses to Ag in adoptive transfer.

The 4-1BB ligand in vitro with agonist Ab, or 4-1BBL expressed artificially, can promote CD4 T cell responses, including proliferation, cytokine secretion, and survival, suggesting that 4-1BB can be costimulatory for CD4 T cells (12, 13). However, results from 4-1BBL/−/− mice have suggested that 4-1BB ligation may not be necessary to generate CD4 T cell responses especially against viral infections (16, 17). To determine the physiological role of 4-1BB in CD4 T cell responses in vivo, we immunized 4-1BB/−/− mice with OVA and addressed the priming of effector CD4 T cells. OVA was administered either with CFA or with Alum to elicit Th1 and Th2 effector responses, respectively. Surprisingly, regardless of the adoptant, 4-1BB/−/− mice generated higher CD4 T cell responses than wild-type mice. The 4-1BB/−/− mice showed enhanced OVA-specific Th1 and Th2 responses in the spleen, which were determined by ELISPOT assays 8 days after immunization.

**FIGURE 2.** Enhanced in vitro recall responses after immunization of 4-1BB/−/− mice. Splenocytes from wild-type and 4-1BB/−/− mice were restimulated with various doses of OVA protein at day 8 following immunization with OVA in CFA (A, C, and E) or OVA in Alum (B, D, and F). A and B, Proliferation at 72 h. C and D, IFN-γ production at 48 h. E and F, IL-4 production at 48 h. Open circles represent wild-type and 4-1BB/−/− mice, respectively. Results are means ± SEM of three mice from triplicate cultures. Similar results were seen in two different experiments.
Enhanced CD4 T cell responses in 4-1BB−/− mice were confirmed by measuring recall responses of splenocytes stimulated with OVA in vitro. Splenocytes from 4-1BB−/− mice showed both enhanced proliferation and cytokine secretion after OVA re-stimulation (Fig. 2). The pattern of cytokine secretion was dependent upon the adjuvant, with splenocytes from mice immunized with OVA in CFA secreting high levels of IFN-γ and splenocytes from mice immunized with OVA in Alum secreting IL-4 (Fig. 2, C and F). Similar to the results with ELISPOT, splenocytes from 4-1BB−/− mice primed with OVA in Alum also secreted a high level of IFN-γ, which was not evident in cell cultures from wild-type mice (Fig. 2D). These results demonstrate that 4-1BB−/− mice are not compromised in being able to prime CD4 T cells, but rather generated enhanced Th1 and Th2 effector responses when immunized with protein Ag, and Th1 responses could be induced in 4-1BB−/− mice that were normally absent in wild-type mice. The 4-1BB−/− mice generate normal Ab responses to protein Ag even though T cell priming is augmented

The 4-1BB agonistic Ab treatment has been shown to abrogate T cell-dependent humoral responses (25), and 4-1BB transgenic mice also exhibited defective Ab responses (24). However, 4-1BB−/− mice generated normal Ab responses following virus infections such as with lymphocytic choriomeningitis virus and influenza (16, 17). To determine whether exaggerated Ab responses accompanied the enhanced CD4 responses we observed, OVA-specific IgM, IgG1, and IgG2c were assessed at 32 days after immunization (Fig. 3). At this time, IgG responses were stronger than IgM, and only immunization in CFA exclusively generated IgG2c, which is a Th1-specific isotype in C57BL/6 mice (26). Both wild-type and 4-1BB−/− mice produced similar levels of IgM and IgG1, although IgG2c responses were reduced in some 4-1BB−/− mice. These results demonstrate that 4-1BB−/− mice are largely similar to wild-type mice in Ab responses even though enhanced frequencies of OVA-reactive T cells were induced.

**The 4-1BB−/− CD4 T cells display enhanced division and clonal expansion in vivo**

Because 4-1BB can be expressed on a number of cell types, including T cells, we wanted to determine whether enhanced CD4 responsiveness was T cell intrinsic or possibly due to the lack of 4-1BB on other cell types that could regulate T cell priming. We therefore generated OT-II TCR transgenic mice on the 4-1BB−/− background and then adoptively transferred 4-1BB−/− CD4 T cells into wild-type (4-1BB-expressing) mice. First, we determined whether wild-type OT-II CD4 T cells expressed 4-1BB under the priming conditions used. The 4-1BB expression was detected on OT-II cells in draining LN at 18 h after immunization with OVA in adjuvant (Fig. 4, A and B), and the level of expression was increased at 70 h (Fig. 4, C and D). Although both regimens induced 4-1BB on OT-II cells, immunization with CFA induced higher expression. These levels were similar to those previously described for T cells in vivo (27).

Previous reports showed that 4-1BB ligation enhanced cell cycle progression of CD4 T cells in vitro (12, 13). To compare early cell division between wild-type and 4-1BB−/− OT-II cells, transferred cells were labeled with CFSE, and dilution of this dye was determined at day 3 after immunization. Unexpectedly, 4-1BB−/− CD4 cells underwent stronger division than wild-type cells. This occurred under both priming conditions, although it was more apparent after immunization with OVA in Alum (Fig. 4, E and F). Next, we determined the overall level of expansion of wild-type and 4-1BB−/− OT-II cells by tracking based on expression of CD4/Vα2/Vß5 and high levels of CD44 to identify activated TCR transgene-positive cells. There was no difference in the homing of naive 4-1BB−/− OT-II cells to spleen or LN after adoptive transfer (data not shown). The number of 4-1BB−/− OT-II cells generated was ~2-fold and 3-fold higher than that of wild-type OT-II cells at day 5 after immunization with OVA

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**FIGURE 3.** Normal Ab responses after immunization of 4-1BB−/− mice. Sera were taken at day 32 after immunization of wild-type and 4-1BB−/− mice. OVA-specific responses were measured by serially diluting sera in combination with HRP-conjugated Abs to individual Ig isotypes. The lowest dilution of sera was 1/200, and sera were serially diluted 3-fold. Each line represents data from one mouse.
in CFA and OVA in Alum, respectively (Fig. 5, A and B). The difference in cell numbers remained constant through to day 8, although absolute numbers decreased due to contraction (average number after immunization with OVA in Alum, OT-II, $2.54 \times 10^5$ vs 4-1BB−/− OT-II, $5.28 \times 10^5$; OVA in CFA, OT-II, $3.21 \times 10^5$ vs 4-1BB−/− OT-II, $6.54 \times 10^5$).

We also examined in vivo apoptosis between 4-1BB−/− and wild-type OT-II cells using annexin V staining, but did not find any significant difference at days 5 and 8 (data not shown), suggesting that the difference in cell accumulation observed was related to increased division. When effector responses of wild-type and 4-1BB−/− OT-II cells were examined by recall assay in vitro after stimulation with OVA$_{323-339}$ peptide, splenocytes from mice receiving 4-1BB−/− OT-II cells displayed significantly enhanced proliferative responses (Fig. 5, C and D) and production of IL-2 (Fig. 5, E and F). Overall, these enhanced responses were directly proportional to the number of OVA-induced CD4 T cells in those mice. Taken together, these results suggest that the absence of 4-1BB expression on CD4 T cells in vivo resulted in a significantly enhanced ability to clonally expand to protein Ag in adjuvant, characterized by a greater propensity to proceed through multiple cell divisions.

The 4-1BB−/− CD4 T cells can display normal responsiveness to Ag

To assess whether 4-1BB−/− CD4 T cells had intrinsic hyperresponsiveness, we stimulated wild-type and 4-1BB−/− OT-II cells with OVA$_{323-339}$ peptide-loaded splenic DC. The 4-1BB−/− OT-II cells normally expressed activation markers such as CD44, CD25, and OX40, but not 4-1BB (data not shown). Moreover, 4-1BB−/− OT-II cells displayed in vitro responses, such as cell...
division (Fig. 6A), proliferation (Fig. 6B), and cell survival/expansion over time (Fig. 6C), which were equivalent to those of wild-type cells. Similarly, when stimulated with PMA and ionomycin, 4-1BB−/− OT-II cells proliferated in an identical manner to wild-type cells (data not shown). These results demonstrate that CD4 T cells from 4-1BB−/− mice are not intrinsically hyperresponsive due to a developmental abnormality, and suggest that the in vivo exaggerated response in the absence of 4-1BB reflects a true unappreciated negative regulatory role for 4-1BB in CD4 priming.

Discussion

Since 4-1BB was identified as a T cell costimulatory molecule, a number of studies have shown that the interaction between 4-1BB and 4-1BBL can enhance T cell responses in vitro and in vivo (reviewed in Refs. 2, 3, and 9). Studies with 4-1BB−/− mice have also supported the notion of a positive regulatory role of 4-1BB in CD8 and some CD4 T cell responses (16, 17, 27, 28). However, the role of 4-1BB in vivo appears to be quite complex, potentially on several levels. For example, 4-1BB agonistic Ab treatment unexpectedly has been shown to inhibit immune responses in several autoimmune disease models (18–21), even though in other cases it can lead to augmented CTL generation and improved immunity against tumors and viruses (29–32). In this study, we now demonstrate another facet of 4-1BB biology, in that the absence of 4-1BB in vivo led to the generation of enhanced Th1 and Th2 effector CD4 T cell responses following immunization with a model protein Ag in several adenoviruses. Moreover, this did not result from a developmental defect, and was at least in part explained by a direct requirement for 4-1BB to be expressed on the responding CD4 cells, which limited their clonal expansion. Our results strongly suggest that physiologically 4-1BB can play a negative regulatory role in CD4 T cell responses in addition to its apparent positive role.

Our results complement and extend data gained from the initial description of 4-1BB-deficient mice, in which it was shown that 4-1BB−/− splenocytes were hyperresponsive with regard to proliferation when stimulated in vitro with plate-coated anti-CD3 (22). We now show that enhanced responses in vivo in 4-1BB−/− mice appear to stem from the hyperresponsiveness of 4-1BB-deficient CD4 T cells because adoptively transferred 4-1BB−/− OT-II cells accumulated to a greater extent than wild-type OT-II cells when responding in a 4-1BB-deficient environment. At least part of the action can be explained by a faster rate of division when 4-1BB cannot be expressed by the responding T cells. There was a possibility that CD4 T cells derived in a 4-1BB-deficient environment possessed an intrinsic hyperresponsiveness to Ag/MHC stimulation, leading to the conclusion that our observations in vivo were developmentally associated. However, the in vitro analyses showed that under standard conditions of Ag presentation, 4-1BB−/− CD4 cells could respond and proliferate equivalently to wild-type cells, arguing against such intrinsic hyperresponsiveness.

What then might account for an augmented T cell response when 4-1BB cannot be expressed? One possibility is the absence of self-regulating signals that might be delivered through the interaction with 4-1BBL, either transmitting a negative signal to the T cell or a negative or modulating signal to the APC-bearing 4-1BBL. However, this idea is questionable because 4-1BBL−/− mice have not shown any apparent signs of enhanced T cell responses in vivo. Rather, the data derived from studies of viral infection have demonstrated reduced T cell priming, consistent with 4-1BB/4-1BBL interactions being costimulatory (16, 17). Furthermore, a recent study using highly complementary systems to those described in this work showed that wild-type OT-II CD4 cells when responding in 4-1BBL-deficient mice following immunization with OVA in LPS were actually impaired in dividing and expanding, again consistent with 4-1BBL positively regulating T cells (27).

There are several other possibilities that might account for enhanced T cell responses in 4-1BB-deficient mice. First, recent reports have shown that 4-1BB is constitutively expressed on some DC (7, 8) and CD4+CD25+ regulatory T cells (33), and it is possible that 4-1BB might negatively modulate these cells, then leading to an increased overall suppressive effect. Second, two reports have shown that 4-1BB ligation with agonist Ab can generate CD8 effector T cells that are regulatory and can suppress CD4 T cell responses, either through production of TGF-β (34), or IFN-γ and subsequent induction of idoleamine 2,3-dioxygenase in CD11b+CD11c+ cells (21). Thus, it is also possible that 4-1BB-controlled CD8 T cells constitutively regulate Ag-induced responses, and in the absence of 4-1BB these cells would be lost, leading to enhanced Ag responsiveness. It certainly is feasible that these types of negative regulation in the intact animal modulate CD4 priming, and hence might have played a role when immunizing 4-1BB-deficient mice.

Another, possibly distinct, action might have accounted for the fact that 4-1BB-deficient CD4 T cells responded more strongly in a wild-type environment in which 4-1BB could be normally expressed. This stems from recent observations made with another TNFR family molecule herpes simplex virus entry mediator (HVEM). The major ligand for HVEM has been thought to be LIGHT (a cellular ligand for HVEM and lymphotixin receptor), and a number of studies have suggested that the LIGHT-HVEM interaction is costimulatory for T cell responses (35, 36). However,

![FIGURE 6.](https://www.jimmunol.org/content/181/11/6807/F6.large.jpg) The 4-1BB-deficient CD4 cells are not intrinsically hyperresponsive and can respond normally in vitro. Wild-type or 4-1BB−/− OT-II CD4 cells were stimulated by coculture with splenic DC prepulsed with various doses of OVA323–339 peptide. A, CFSE dilution profiles of responding T cells (gated on CD4 and Vα2) at day 3. B, Proliferation at 72 h. C, Kinetics of T cell expansion/survival. Recovery is shown as a percentage of input cell number (gated on CD4 and Vα2). A and C, T cells were stimulated with 1 µM peptide-loaded DC. Wild-type T cells, open symbols. The 4-1BB−/− T cells, closed symbols. Data are representative of three experiments.
recent data with HVEM-deficient cells have also demonstrated hyperresponsiveness (K. Potter and C. Ware, unpublished observations), somewhat similar to the phenotype we describe in this study with 4-1BB. HVEM has now been shown to also bind the ITIM-containing molecule B and T lymphocyte attenuator (BTLA) that is expressed on both activated T cells and APC, and can transmit a negative signal, at least in part by recruiting the phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-2) (37, 38). This is the first example of an Ig superfamily member binding a TNFR family member, and in many respects resembles the interactions of B7-1/B7-2 with CD28 or CTLA4 (39). Thus, there are at least two examples of receptors that can bind both stimulatory and inhibitory ligands. It is an intriguing notion that the hyperresponsiveness of 4-1BB-deficient T cells reported in this work is explained through 4-1BB possessing another as yet undiscovered ligand that might provide a negative modulatory influence similar to BTLA and CTLA4.

In summary, we have provided new and novel data to indicate that 4-1BB can play a modulatory role on CD4 T cells distinct from its described action as a costimulatory molecule. This results in suppressing CD4 T cell proliferation and limiting the extent of clonal expansion to Ag. In the future, it will be important to understand how this action is brought about to fully realize the influence of 4-1BB on CD4 T cell priming and the extent to which 4-1BB biology contributes to immune function. Moreover, it remains to be assessed whether the absence of 4-1BB on CD8 T cells also leads to hyperresponsiveness, or whether fundamental differences exist between the control of CD8 vs CD4 T cells.

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Disclosures
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