The Journal of Immunology

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J Immunol 2015; 194:134-141; Prepublished online 17 November 2014;
doi: 10.4049/jimmunol.1401383
http://www.jimmunol.org/content/194/1/134

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

4-1BB Ligand Signaling to T Cells Limits T Cell Activation

So-Young Eun,* Seung-Woo Lee,† Yanfei Xu,* and Michael Croft*

4-1BB ligand (4-1BBL) and its receptor, 4-1BB, are both induced on T cells after activation, but little is known about the role of 4-1BBL. In this study we show that 4-1BBL can transmit signals that limit T cell effector activity under tolerogenic conditions. Cross-linking 4-1BBL inhibited IL-2 production in vitro, primarily with suboptimal TCR stimulation. Furthermore, naive 4-1BBL–deficient OT-II transgenic T cells displayed a greater conversion to effector T cells in vivo when responding to soluble OVA peptide in wild-type hosts, whereas development of Foxp3$^+$ regulatory T cells was not altered. A greater number of effector T cells also differentiated from naive wild-type OT-II T cells when transferred into 4-1BB–deficient hosts, suggesting that APC-derived 4-1BB is likely to trigger 4-1BBL. Indeed, effector T cells that could not express 4-1BBL accumulated in larger numbers in vitro when stimulated with 4-1BB–expressing mesenteric lymph node dendritic cells. 4-1BBL was expressed on T cells when Ag presentation was limiting, and 4-1BBL was aberrantly expressed at very high levels on T cells that could not express 4-1BB. *Trans*-ligation, Ab capture, and endocytosis experiments additionally showed that T cell–intrinsic 4-1BB regulated internalization of membrane 4-1BBL, implying that the strong induction of 4-1BB on T cells may counteract the suppressive function of 4-1BBL by limiting its availability. These data suggest that 4-1BBL expressed on T cells can restrain effector T cell development, creating a more favorable regulatory T cell to effector cell balance under tolerogenic conditions, and this may be particularly active in mucosal barrier tissues where 4-1BB–expressing regulatory dendritic cells present Ag. *The Journal of Immunology*, 2015, 194: 134–141.

Tumor necrosis factor and TNFR superfamily interactions play crucial roles in regulating proinflammatory as well as anti-inflammatory responses in autoimmune diseases (1, 2). Among these, the binding of 4-1BB to 4-1BB ligand (4-1BBL) has been documented to promote cell activation, survival, and differentiation, primarily through 4-1BB signaling activity in T cells, NK cells, and dendritic cells (DCs). However, there have been reports that 4-1BB–deficient T cells and myeloid lineage cells hyperproliferate (3, 4), suggesting that the interaction between 4-1BB and 4-1BBL might also be suppressive in certain situations. This suppressive action may be transmitted through 4-1BB itself, leading to production of modulatory molecules such as retinaldehyde dehydrogenase (RALDH) in DCs as recently described (5). However, such suppressive function may also be attributed in alternate scenarios to signals emanating from 4-1BBL. Although 4-1BBL was originally thought to be simply a ligand for 4-1BB (6), there has been accumulating evidence that it can transduce signals when interacting with 4-1BB (7, 8). Cross-linking 4-1BBL was shown to promote or suppress immune cell differentiation, suggesting that the result of 4-1BBL signaling is likely to be cell-specific and/or context-dependent (4, 9–11). In particular, ligation of 4-1BBL with an Fc fusion protein of 4-1BB promoted IL-10 production in bone marrow–derived macrophages, supporting its potential suppressive functionality (10), and several studies with 4-1BB–Fc or 4-1BB–expressing cells have reported inhibition of T cell responsiveness, implying a negative activity of 4-1BBL (3, 12, 13). Despite some reports finding evidence of expression of 4-1BBL on activated T cells (6, 14), 4-1BBL is usually barely detectable, and thus the regulation and primary function of 4-1BBL on this cell type is still not clear.

In this study, we demonstrate a T cell–intrinsic regulation of 4-1BBL by 4-1BB itself when 4-1BB is strongly induced in a T cell. We find that 4-1BBL signaling can play a very early rate-limiting step in Ag-dependent T cell activation, under conditions of limiting Ag presentation and inflammation, when 4-1BB is not highly expressed, suppressing IL-2 production and effector T cell clonal expansion. We also show that the physiological relevance of 4-1BBL–mediated suppressive function in T cells may manifest in microenvironments such as the GALT where 4-1BB–expressing DCs may encounter recently activated T cells that express 4-1BBL, further adding to the ability of regulatory APCs to limit the differentiation or expansion of effector T cells.

Materials and Methods

Mice

Eight- to 10-wk-old C57BL/6 (B6) mice or Ly5.1 congenic B6 mice were purchased from The Jackson Laboratory. 4-1BB$^{-/-}$ or 4-1BBL$^{-/-}$ OT-II TCR transgenic mice were generated by crossing OT-II mice with 4-1BB$^{-/-}$ mice (from B.S. Kwon) (3) or 4-1BBL$^{-/-}$ mice (from Amgen), respectively, and maintained at La Jolla Institute for Allergy and Immunology. All experiments were done in compliance with the regulations of the La Jolla Institute for Allergy and Immunology Animal Care Committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

Murine 4-1BB and 4-1BBL constructs

Murine 4-1BB cDNA was synthesized by RT-PCR using total RNA from activated splenic CD4 T cells of B6 mice. Full-length (256 aa) and cytoplasmic tail-deleted (ΔC, 213 aa) 4-1BB expression constructs were generated in pBABE-puro backbone (a gift of Dr. Chris Benedict, La Jolla Institute for Allergy and Immunology) tagged with myc (5′-GAGCAGAAGCTGATCAGCGAGGAAGACCTG-3′, EQKLISEEDL) in the N terminus and His6 (5′-CACCATCACCATCACCAT-3′, HHHHHH) in the

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Received for publication May 30, 2014. Accepted for publication October 21, 2014.

This work was supported by National Institutes of Health Grants AI042944 and AI089624 (to M.C.). This is manuscript 1729 from the La Jolla Institute for Allergy and Immunology.

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Abbreviations used in this article: B6, C57BL/6; 4-1BBL, 4-1BB ligand; DC, dendritic cell; MLN, mesenteric lymph node; RALDH, retinaldehyde dehydrogenase; Treg, regulatory T; WT, wild-type.

C terminus. Murine 4-1BBL cDNA was obtained from total RNA of splenic CD11c$^+$ cells by RT-PCR. 4-1BBL (full length of 309 aa) was also inserted into pBABE-puro with an N-terminal FLAG tag (5′-GACTACAAGGACGATGACGATAAG-3′, MDYKDDDDK) and a C-terminal hemagglutinin tag (5′-TACCCTTATGACGTGCCAGATTACGCC-3′, YPYDVPDYA).

Cells and in vitro cultures

CD4$^+$ T cells were enriched from the spleen and lymph nodes of wild-type (WT), 4-1BB$^{-/-}$, or 4-1BBL$^{-/-}$ mice on the OT-II TCR transgenic background by using a CD4 T cell isolation kit with LS columns (Miltenyi Biotec), according to the manufacturer's instructions. Naive (CD44^{lo}CD62L^{hi}CD25$^-$) CD4$^+$ T cells were further purified by FACS. T cells (2×10^5/200 μl) were cultured either with DCs ($1–4 \times 10^4$) and various amounts of $OVA_{323–339}$ peptide Ag or with variable concentrations of immobilized anti-CD3 (2C11) and 2.5 μg/ml soluble anti-CD28 (37N5) in each well of 96-well plates at 37°C with 5% CO_2. In some experiments, rat anti-mouse 4-1BB (3H3, originally from Dr. Robert Mittler, Emory University), rat anti-mouse 4-1BBL (19H3, also from Dr. Robert Mittler), or 4-1BB–Fc (15) were added to the culture in soluble or immobilized forms (20 μg/ml). Rat IgG (Sigma-Aldrich) and human IgG1 Fc (Millipore) were used as controls and added soluble or immobilized in the exact manner as the 4-1BB/4-1BBL reagents. For endocytosis inhibition assays, chlorpromazine or genistein (Sigma-Aldrich) was added to the culture for the final 6 h. For regulatory T (Treg) cell conversion experiments, 4-1BB–expressing CD11c$^+$MHC class II^{hi} DCs from mesenteric lymph nodes (MLNs) were pre-enriched by removing T/B lymphocytes, NK cells, and γδT cells using biotinylated anti-CD3ε, anti-B220, anti-DX5α, and anti-γδTCR (eBioscience) along with the EasySep Mouse Streptavidin RapidSpheres Isolation Kit (StemCell Technologies) according to the manufacturers' instructions, and then further sorted by FACS using FITC-conjugated anti-CD11c, Pacific Blue–conjugated anti–I-A/I-E (BioLegend), biotinylated anti–4-1BB with allophycocyanin-conjugated streptavidin (eBioscience). Recombinant human TGF-β1 (R&D Systems) was used for Treg cell conversion assays. In some cases, purified naive CD4 T cells were labeled with 0.5 μM CFSE (Invitrogen).

T hybridoma cells were generated from activated 4-1BB$^{-/-}$ OT-II T cells fused with the BW5147 thymoma, and selected based on production of IL-2. Myc-tagged full-length or cytoplasmic region–deleted 4-1BB vectors were packaged in HEK293T cells, and supernatants were used to introduce the constructs into the T hybridoma cells by centrifugation at 3000 rpm for 2 h at room temperature. 4-1BB–expressing hybridoma cells were selected with 0.75 μM puromycin treatment and purified by FACS based on Myc tag expression. In experiments, 1×10^5 cells were cocultured with mock-transfected T hybridoma cells (1×10^5) in 200 μl volumes for 24 h. To stimulate 4-1BBL via cell-to-cell interaction, recombinant 4-1BB was introduced into 3T3 fibroblasts or thymoma cells by retroviral transduction as described above, and used as 4-1BB–expressing accessory cells along with 4-1BB$^-$ mock cells as controls.

Adoptive transfer, Ag administration, and ex vivo analysis

Purified naive WT or 4-1BBL$^{-/-}$ OT-II T cells (2×10^6) were i.v. transferred into congenic Ly5.1 B6 mice. In some experiments, WT and 4-1BB$^{-/-}$ B6 mice were used as recipients of WT Ly5.1$^+$ congenic OT-II T cells. Twenty-four hours after transfer, 5 μg $OVA_{323–339}$ peptide was injected in PBS into each mouse through the tail vein. At day 3, 6, or 9 after peptide administration, spleens and lymph nodes were analyzed by flow cytometry for accumulation of OT-II T cells (Vα2$^+$Vβ5$^+$Ly5.2$^+$) with an effector phenotype (CD44^{hi}CD62L^{lo}) or Treg phenotype (Foxp3$^+$). Splenocytes, harvested at day 3, were also restimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml), and culture supernatants were assayed for production of IL-2 and IFN-γ by ELISA. In an alternate protocol, soluble OVA peptide was injected 24 h after adoptive transfer (25 μg) and then again 4 d later (10 μg). Transferred OT-II T cells from spleens and lymph nodes were then analyzed 3 d after the second challenge (day 7) by flow cytometry.

Flow cytometry

For 4-1BB and 4-1BBL detection, biotinylated anti–4-1BB (17B5) and anti–4-1BBL (TKS-1) Abs were used, respectively, along with biotinylated rat IgG2a as an isotype control, plus streptavidin-conjugated allophycocyanin (all from eBioscience). For analyzing activated T cells, anti–CD44-FITC, anti–CD62L-allophycocyanin, anti–Vα2-Pacific Blue, anti–Vβ5-PE, and anti–CD4-allophycocyanin-Cy7 were used, along with anti-CD45.1 or anti–CD45.2-PerCP-Cy5.5. For detection of Foxp3$^+$ Treg cells, anti–Foxp3-PerCP-Cy5.5 was used along with other T cell markers including anti–CD25-PE-Cy7 (all from eBioscience).

ELISA

Unlabeled anti-mouse IL-2 (0.5 μg/ml) or anti-mouse IFN-γ (0.5 μg/ml), biotinylated anti-mouse IL-2 or IFN-γ, and streptavidin-conjugated HRP (BD Biosciences) were used in conventional ELISA assays to assess IL-2 or IFN-γ secretion using tetramethylbenzidine substrate reagents (BioLegend). The OD was read at 450 nm on a SpectraMax 250 (Molecular Devices). The amount of IL-2 or IFN-γ in each sample was determined based on the standard curve generated with serially diluted recombinant murine IL-2 or IFN-γ (PeproTech).

Immunofluorescence microscopy

Activated T cells (2×10^5) were loaded onto each poly-L-lysine–coated coverslip (BD Biosciences), fixed with 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.3% saponin/PBS for 5 min, treated with 5% BSA/PBS for 30 min, and stained with anti–4-1BBL goat pAb (R&D Systems) and biotinylated anti–4-1BB (3E1) mAb (biotinylated using 3E1 Ab, a gift from Dr. Robert Mittler, Emory University) for 1 h, followed by donkey anti-goat IgG (H+L)–Alexa Fluor 488 and streptavidin–Alexa Fluor 647 (Invitrogen) for another hour. Cells were washed with PBS three times between the steps. Cellular nuclei were revealed with DAPI staining. Immunofluorescent images were obtained under Zeiss Axiovert 200M microscope integrated with Intelligent Imaging Innovations slidebook 4.2 and analyzed using ImageJ software.

Statistical analysis

All statistical analyses were performed using a two-tailed Student *t* test.

Results

4-1BBL is induced on activated T cells but downregulated when 4-1BB is strongly expressed

Certain TNF family ligands have been characterized as primarily expressed on T cells, notably molecules such as CD40L and LIGHT. However, it is not clear whether ligands for many of the TNF family receptors that are costimulatory for T cells, such as 4-1BB, OX40, GITR, and CD27, are also ubiquitously present, and what might regulate their availability. Understanding this is also complicated by the fact that many TNF family ligands might be cleaved from the cell membrane or regulated by endocytosis. 4-1BBL is normally hard to detect on T lymphocytes, but we found that 4-1BB–deficient T cells displayed high levels of 4-1BBL on their surface upon activation (Fig. 1A). This type of regulation is consistent with previous studies that revealed higher levels of 4-1BBL on hematopoietic stem cells and DC precursors when obtained from 4-1BB–deficient animals (4). Suggesting this was not related to abnormal activity of 4-1BB–deficient T cells, we also found that an anti–4-1BBL Ab that can block the interaction of 4-1BB with 4-1BBL also revealed strong expression of surface 4-1BBL by flow cytometry and in confocal experiments when added in soluble form into WT T cell cultures (Fig. 1B, 1C). Anti–4-1BBL may have simply captured 4-1BBL on the cell surface; however, Abs directed to 4-1BB also revealed surface 4-1BBL (data not shown), suggesting that an interaction between the two molecules reduces the amount of 4-1BBL present on the T cell surface. We also found that intracellular levels of 4-1BBL were higher in 4-1BB$^{-/-}$ than in WT T cells, as well as in WT T cells cultured in the presence of anti–4-1BBL (Fig. 1D). 4-1BBL became detectable on the cell surface after 15 min at low levels and was highly expressed between 24 and 48 h upon addition of soluble anti–4-1BBL, correlating with the normal induction kinetics of the receptor (data not shown and Fig. 1). This suggests that 4-1BBL is produced and potentially available to play a functional role after T cells are activated, but when 4-1BB is made at high levels, it can limit the expression of its own ligand.

To further study this, a T cell hybridoma was generated from 4-1BB–deficient primary activated T cells and then retrovirally transduced with 4-1BB. Parent T hybridoma cells endogenously expressed surface 4-1BBL due to the lack of 4-1BB, and trans-

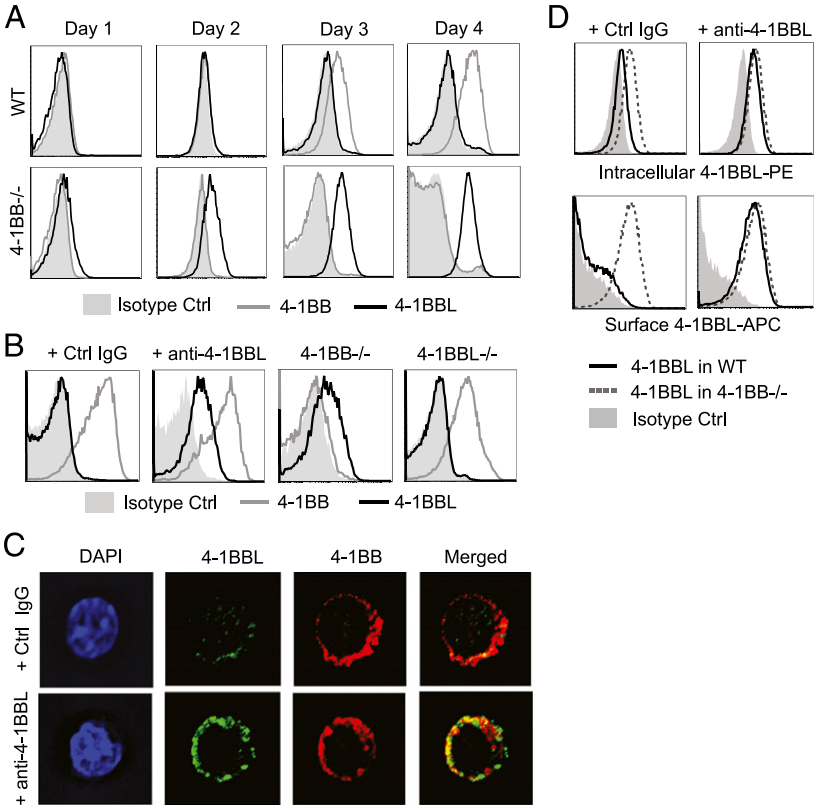

FIGURE 1. Expression of 4-1BB and 4-1BBL on activated T cells. WT, 4-1BB$^{-/-}$, and 4-1BBL$^{-/-}$ naive OT-II CD4 T cells were either activated with WT DCs and 1 μM OVA peptide for varying lengths of time (**A**) or anti-CD3 and anti-CD28 in the presence of soluble anti–4-1BBL (19H3) or control IgG for 48 h (**B**–**D**). (A and B) 4-1BB and 4-1BBL surface expression in gated CD44^{hi}Vα2^{+}Vβ5^{+} T cells by flow cytometry. (C) 4-1BB and 4-1BBL expression in T cells from (B) analyzed by confocal microscopy. Green, 4-1BBL; red, 4-1BB. Original magnification ×80. (D) Intracellular staining for 4-1BBL (*top*) after surface 4-1BBL was stained with saturating amounts of the same detection Ab conjugated to a different dye. Data are representative of three to five independent experiments.

duction with mock vector did not alter this endogenous expression (Fig. 2A). In contrast, transduction with full-length 4-1BB resulted in strongly diminished the levels of surface 4-1BBL. Introduction of a cytoplasmic domain–deleted mutant form of 4-1BB (ΔC 4-1BB) also reduced surface 4-1BBL to a similar degree. This showed that the cytoplasmic tail of 4-1BB and signaling through 4-1BB are dispensable for this activity, and again suggested that the reduction of expression of 4-1BBL requires an interaction with 4-1BB. To then determine whether 4-1BB could suppress surface 4-1BBL expressed on a neighboring cell (in *trans*), mock-transduced cells expressing endogenous 4-1BBL were cocultured at 1:1 ratio for 24 h with cells transduced with full-length or ΔC 4-1BB. 4-1BB expressed in *trans* did not alter the level of 4-1BBL expressed on the mock cells (Fig. 2B), indicating that T cell–intrinsic expression of 4-1BB limits 4-1BBL levels in the same cell through a *cis* interaction, either intracellularly or on the membrane.

We then performed assays to block endocytosis to determine whether this is one of the mechanisms by which 4-1BBL expression is controlled on the T cell surface (Fig. 3). A 6-h treatment of WT T cells with genistein, an inhibitor of caveolin-mediated endocytosis, resulted in expression of surface 4-1BBL at similar levels to those detectable on 4-1BB–deficient T cells. In contrast, an inhibitor of clathrin-mediated endocytosis, chlorpromazin, had little effect on the expression of 4-1BBL. Quantitative PCR analysis from activated WT and 4-1BB$^{-/-}$ T cells showed no difference in expression of mRNA for 4-1BBL (data not shown), suggesting that the dominant mechanism for 4-1BB–facilitated downregulation of 4-1BBL is posttranscriptional. Because intracellular levels of 4-1BBL were also decreased in WT T cells compared with 4-1BB–deficient T cells, or in WT T cells treated with anti–4-1BBL blocking Ab (Fig. 1D), these data then suggest that 4-1BBL protein is degraded after endocytosis triggered by 4-1BB.

4-1BBL signaling limits T cell activation in vitro

As there was a reciprocal relationship between expression of 4-1BBL and its receptor, this implied that 4-1BBL might be functionally active in activated T cells in certain situations where 4-1BB might not be optimally induced. Thus, we first tested whether signaling through 4-1BBL, brought about by cross-linking with immobilized anti–4-1BBL or an Fc fusion of 4-1BB, would have any effect on the response of T cells to TCR stimulation with or without costimulation through CD28. Significantly, T cell activation to varying doses of anti-CD3 with anti-CD28 was suppressed in the presence of immobilized anti–4-1BBL, as determined by assessing the levels of secreted IL-2 (Fig. 4A). Notably, this

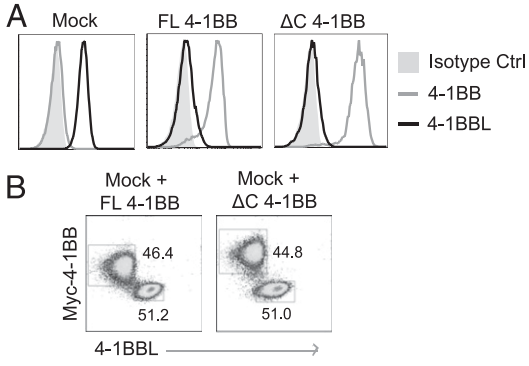

FIGURE 2. T cell–intrinsic suppression of 4-1BBL expression by 4-1BB. (**A**) 4-1BB–deficient CD4 T hybridoma cells mock transfected or transfected with Myc-tagged full length (FL) 4-1BB or C term–deleted mutant (ΔC) 4-1BB were stained for membrane 4-1BB and 4-1BBL. (**B**) FL 4-1BB– or ΔC 4-1BB–transfected T hybridoma cells (1×10^5) were cocultured at a 1:1 ratio with mock-transfected T hybridoma cells (expressing endogenous 4-1BBL) for 24 h and stained for membrane 4-1BB and 4-1BBL. All data are representative of two independent experiments.

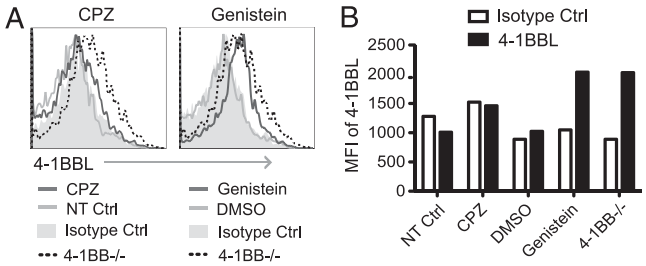

FIGURE 3. 4-1BBL surface expression is regulated by endocytosis. WT naive CD4 T cells were activated with anti-CD3 and anti-CD28. After 42 h, endocytosis blockers, chlorpromazine (CPZ; 10 μg/ml) or genistein (500 μM), were added to the cultures and incubated for an additional 6 h. Controls were nontreated (NT) or DMSO treated. Cells were stained for membrane 4-1BBL. 4-1BB$^{-/-}$ T cells were also cultured as an additional positive control for 4-1BBL expression. (**A**) Flow plots. (**B**) Mean fluorescence intensity (MFI) of 4-1BBL among the different groups. Data are representative of two independent experiments.

activity of binding 4-1BBL was strongest under conditions of suboptimal stimulation with anti-CD3. Varying the dose of anti-CD28 did not alter the suppressive effect (not shown). CFSE dilution assays also showed a significant decrease in the number of cell divisions as well as the total number of dividing cells in the presence of immobilized anti–4-1BBL, with this effect limited to low doses of anti-CD3, correlating with the action on IL-2 production (Fig. 4B and data not shown). To verify that suppression of T cell activation was a direct result of signaling through 4-1BBL and not because of disrupting 4-1BB/4-1BBL interactions, 4-1BB$^{-/-}$ T cells that express higher levels of endogenous 4-1BBL were also tested. Immobilized anti–4-1BBL suppressed IL-2 production to a similar extent in 4-1BB$^{-/-}$ T cells compared with WT T cells (Fig. 4A). An equivalent effect was also seen with immobilized 4-1BB–Fc (Fig. 4C) showing suppression was not Ab specific but could result when 4-1BBL bound its natural partner. Similarly, 4-1BBL–expressing T hybridoma cells derived from activated 4-1BB$^{-/-}$ OT-II cells also produced lower levels of IL-2 when stimulated with anti-CD3 or anti-CD3/anti-CD28 in the presence of immobilized 4-1BB–Fc (Fig. 4D). The suppressive activity was further confirmed by using irradiated accessory cells that expressed 4-1BB to ligate 4-1BBL on these T cells. Reduced IL-2 production was observed compared with T cells cultured with accessory cells that lacked 4-1BB (Fig. 4E). These data collectively suggest that 4-1BBL plays a suppressive role in T cell activation under conditions of suboptimal stimulation. This negative activity is controlled when T cells are stimulated strongly to express high levels of 4-1BB, which in *cis* configuration facilitates the removal of 4-1BBL from the T cell surface by endocytosis.

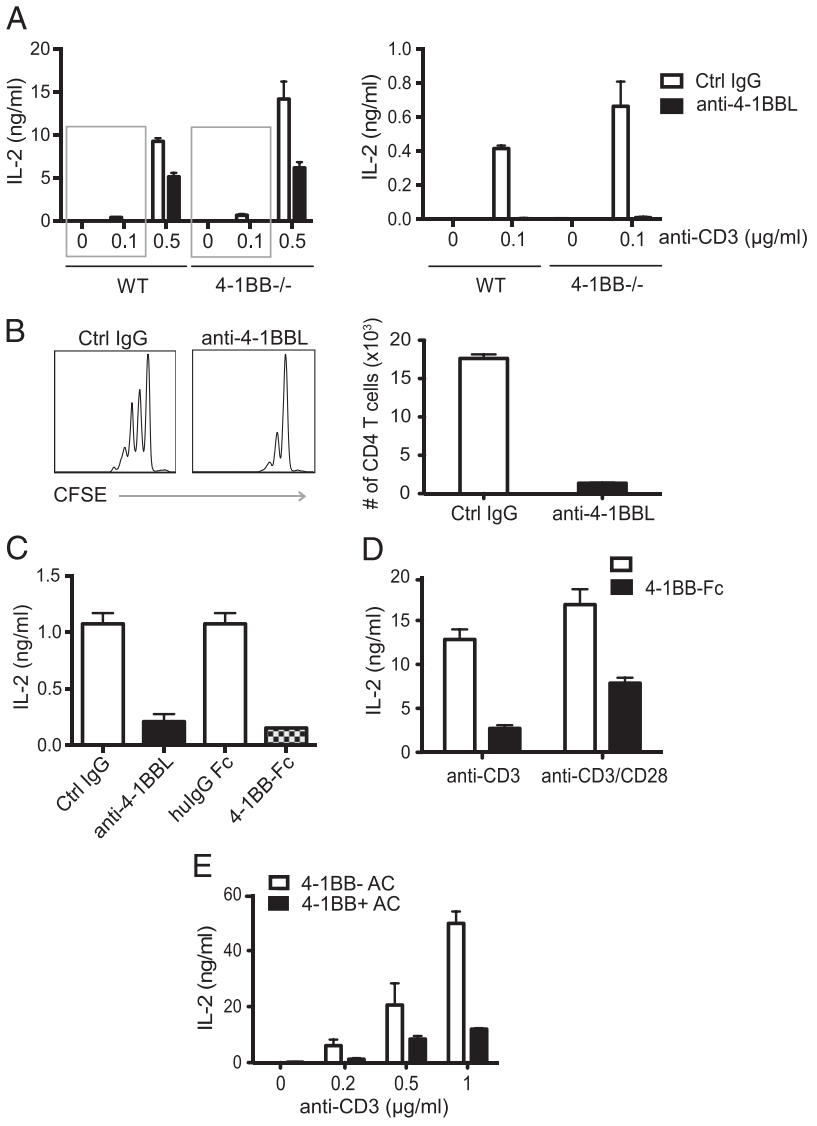

FIGURE 4. T cell activation is suppressed by 4-1BBL signaling. (**A**) WT and 4-1BB$^{-/-}$ naive CD4 T cells were stimulated with various concentrations of anti-CD3 and 2.5 μg/ml anti-CD28 in the presence of plate-bound anti–4-1BBL (20 μg/ml) or control IgG. IL-2 was assessed at 48 h by ELISA. *Right panel* shows data magnified from *left panel* (gray boxes). (**B**) CFSE-labeled naive CD4 T cells were stimulated with 0.1 μg/ml anti-CD3 and 2.5 μg/ml anti-CD28 in the presence of plate-bound anti–4-1BBL or control IgG for 48 h. CFSE dilution was assessed (*left*) and CD4 T cell recovery was calculated (*right*). (**C**) Naive 4-1BB$^{-/-}$ CD4 T cells were stimulated with a low dose of plate-bound anti-CD3 and anti-CD28 as in (A) in the presence of plate-bound anti–4-1BBL or 4-1BB–Fc (20μg/ml), or control rat IgG or human IgG1 Fc. IL-2 was assessed at 48 h by ELISA. (**D**) 4-1BB$^{-/-}$ T hybridoma cells were activated with anti-CD3 (0.1 μg/ml) with or without anti-CD28 (2.5 μg/ml) in the presence of plate-bound 4-1BB–Fc or control human IgG1 Fc (20 μg/ml). IL-2 was assessed at 6 h by ELISA. (**E**) 4-1BB$^{-/-}$ T hybridoma cells were activated with various concentrations of anti-CD3 in the presence of irradiated accessory cells (AC) that did or did not express 4-1BB. IL-2 was assessed at 6 h by ELISA. Data are representative of five independent experiments and are means ± SEM from replicate cultures.

4-1BBL signaling limits effector T cell development in vivo under noninflammatory conditions

To investigate any physiological relevance of these results, we assessed conditions in vivo where peptide was recognized under noninflammatory/tolerogenic conditions that favor development of Foxp3+ Treg cells and that might mimic the scenario we found in vitro where 4-1BBL was actively suppressive in T cells (16). The response of naive TCR transgenic T cells that could or could not express 4-1BBL was tracked when adoptively transferred into WT hosts. With systemic injection of a low dose of OVA peptide Ag in PBS, we found that the absence of 4-1BBL$^{-/-}$ on the responding naive T cells resulted in accumulation of ~3-fold more effector T cells (CD44^{hi}, CD62^{lo}) in spleens or lymph nodes when assessed after 3 d (Fig. 5A, *left*). In contrast, a similar number of Foxp3^{+} OT-II Treg cells developed regardless of the presence or absence of 4-1BBL on the responding T cells (Fig. 5A, *middle*). The enhanced numbers of effector T cells generated in the absence of 4-1BBL was maintained at day 6, although the absolute numbers were reduced compared with day 3 regardless of being WT or 4-1BBL$^{-/-}$ (Fig. 5A, *left*). After 9 d, we could not detect effector T cells regardless of being WT or 4-1BBL$^{-/-}$ (not shown). Consistent with this being a tolerogenic response, Foxp3^{+} Treg cells were maintained over this time period and were similar in number in both groups (not shown). These data suggested that 4-1BBL principally acted to limit the generation of effector T cells as Treg cells were forming to aid in the development of tolerance. In line with this, higher levels of IL-2 and IFN-γ were detected in splenic cultures from mice receiving 4-1BBL$^{-/-}$ T cells (Fig. 5B). To ascertain whether the suppressive activity of 4-1BBL on T cells came from its interaction with 4-1BB expressed in the hosts, presumably on APCs, 4-1BB$^{-/-}$ mice were used as recipients of WT OT-II T cells. Two- to 3-fold higher numbers of OVA-specific T cells of the effector phenotype were generated in 4-1BB$^{-/-}$ recipients, paralleling the observation with 4-1BBL–deficient T cells (Fig. 5C). In contrast, there was no significant difference in the numbers of Foxp3^{+} Treg cells generated in both groups.

To test the effect of 4-1BBL in another system, we challenged mice twice with soluble OVA peptide in PBS, with the second injection given after 4 d, and then assessed the number of effector T cells generated after a further 3 d (7 d total). In this scenario, higher numbers of effector T cells were maintained over this time frame compared with a single peptide injection, but importantly the difference between WT and 4-1BBL$^{-/-}$ T cells was maintained at an ~1:3 ratio (Fig. 5D). Again, Foxp3^{+} Treg cells were generated

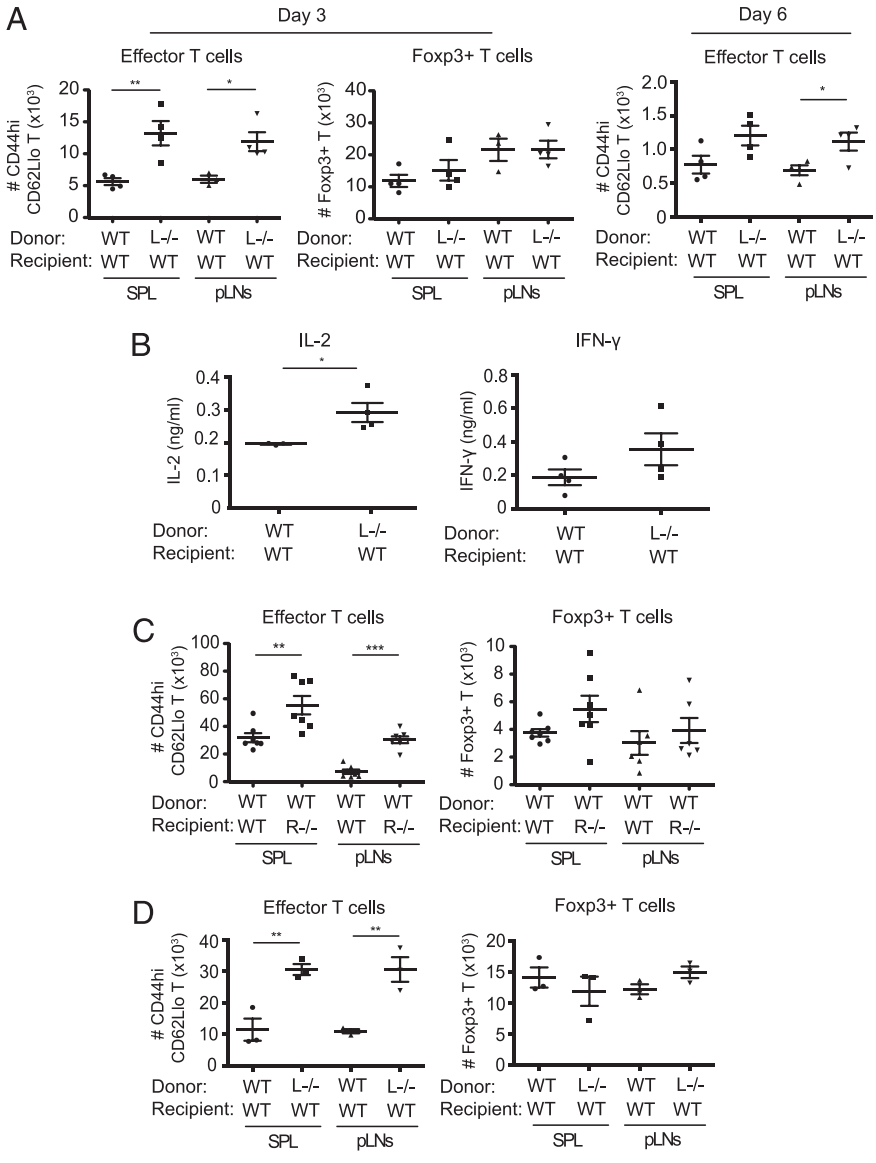

FIGURE 5. 4-1BBL limits T cell activation in vivo under noninflammatory conditions. (**A**) Sorted naive WT or 4-1BBL$^{-/-}$ (L$^{-/-}$) Ly5.2^{+} OT-II T cells (2×10^6) were adoptively transferred into WT Ly5.1^{+} congenic recipient mice. One day later, mice were immunized i.v. with 5 μg $OVA_{323–339}$ peptide in PBS. After 3 d (*left* and *middle*) or 6 d (*right*), the number of effector (CD44^{hi}CD62L^{lo}) or Foxp3^{+} OT-II (Vα2^{+}Vβ5^{+}Ly5.2^{+}) T cells was calculated in spleens (SPL) and peripheral lymph nodes (pLNs). (**B**) Splenocytes from (A), taken at day 3, were stimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 5 h, and IL-2 and IFN-γ production were measured by ELISA. (**C**) Sorted naive Ly5.1^{+} congenic WT OT-II T cells were transferred into WT or 4-1BB$^{-/-}$ (R$^{-/-}$) Ly5.2^{+} recipients. Mice were immunized and analyzed at day 3 as in (A). (**D**) Recipients of 4-1BBL$^{-/-}$ T cells were challenged with 25 μg OVA peptide in PBS similar to (A) and then rechallenged with 10 μg OVA peptide 4 d later. Accumulation of effector and Foxp3^{+} OT-II T cells was analyzed after a further 3 d. All data show numbers of T cells or amounts of each cytokine in individual recipient mice, with means ± SEM for each group, and are representative of at least two independent experiments in each case. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

equally regardless of the absence of 4-1BBL. Furthermore, we observed no significant difference in the response of 4-1BBL–deficient T cells compared with WT T cells when the adjuvant aluminum hydroxide was given along with OVA peptide using a similar immunization protocol that does not generate significant numbers of Treg cells (data not shown). Thus, 4-1BBL expressed on T cells suppresses the initial accumulation and differentiation of effector populations under noninflammatory conditions where Treg cells are also generated, but it has no apparent role in the T cell response under inflammatory conditions.

4-1BB–4-1BBL interactions between regulatory DCs and T cells limits T cell activation

Previously we reported that a proportion of MLN DCs implicated in promoting the generation of Foxp3$^+$ Treg cells constitutively expressed 4-1BB. This is the subset that also expresses CD103 and makes high levels of the regulatory enzyme RALDH that controls retinoic acid production. We furthermore found that 4-1BB participated in the development of this subset of regulatory DCs from precursors by determining the level of expression of RALDH (5). To assess whether 4-1BB on these DCs may also promote suppressive activity by binding T cell–expressed 4-1BBL, WT or 4-1BBL$^{-/-}$ naive OT-II T cells were cocultured with sorted 4-1BB–expressing MLN DCs (CD11c$^+$MHC class II^{hi}4-1BB$^+$). 4-1BBL$^{-/-}$ effector T cells accumulated to a greater extent with a low dose of Ag (Fig. 6A), in line with limiting Ag or inflammation revealing the suppressive effect of 4-1BBL. With a high dose of Ag, 4-1BB was strongly induced on WT T cells whereas its expression was weaker with a low dose of Ag (Fig. 6B). Correspondingly, 4-1BBL was readily detectable with a low dose of Ag, expressed together with 4-1BB, but it was much more weakly detectable with a high dose of Ag when 4-1BB was present at higher levels (Fig. 6B). This likely accounts for why it was functionally relevant under the former conditions.

Despite that 4-1BBL signaling did not alter Foxp3$^+$ Treg cell differentiation in the conditions of immunization we used in vivo (Fig. 5), it remained possible that 4-1BBL signaling might contribute to the proportion of Treg cells that are generated in certain microenvironments such as the GALT where TGF-β is strongly expressed and 4-1BB–expressing DCs are found. To test this in vitro, sorted 4-1BB$^+$ MLN DCs were cultured with 4-1BBL$^{-/-}$ T cells in the presence of TGF-β. A significantly reduced percentage of Foxp3$^+$ Treg cells were generated when 4-1BBL could not be expressed (3.3 versus 9.6%). This was limited to the cultures with a low dose of Ag whereas with a high dose of Ag no defect in Treg cell generation was apparent (Fig. 6C). This again correlated with little detectable 4-1BBL and strong 4-1BB expression under the latter conditions, whereas we again observed with a low dose of Ag that 4-1BBL was more strongly expressed and most of the 4-1BBL–expressing cells also coexpressed 4-1BB (Fig. 6C and data not shown). In the cultures with limiting Ag, there were also many T cells that neither expressed 4-1BB or 4-1BBL, most likely because they were not stimulated well through the TCR. Thus, 4-1BBL can exert suppressive activity in T cells to limit effector cell development under conditions of weak Ag presentation or no inflammation, and in certain conditions 4-1BBL may also participate in allowing greater development of Treg cells by ligating 4-1BB on APCs.

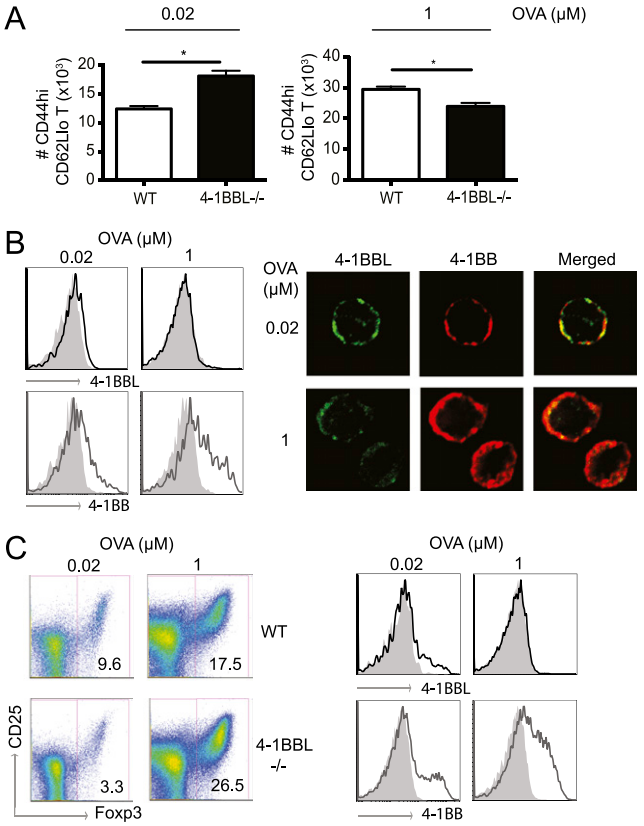

FIGURE 6. 4-1BBL signaling in T cells limits T cell activation and favors Treg cell generation with low-dose Ag. (**A**) Naive WT or 4-1BBL$^{-/-}$ OT-II T cells were cultured with sorted 4-1BB$^+$ MLN DCs (MHC class II^{hi}, CD11c$^+$) at 20:1 ratios with 0.02 or 1 μM OVA peptide for 72 h. Recovery of effector phenotype OT-II T cells (CD44^{hi}CD62L^{lo}) was determined. Data are means ± SEM from triplicate cultures. (**B**) T cells from cultures in (A) were analyzed for expression of 4-1BBL and 4-1BB by flow cytometry (*left*) and confocal microscopy (*right*). Original magnification ×80. Green, 4-1BBL; red, 4-1BB. (**C**) T cells were cultured as in (A) but in the presence of 5 ng/ml TGF-β1. Percentages of Foxp3$^+$ OT-II T cells were determined with costaining for CD25 after 5 d (*left*), and expression of 4-1BBL and 4-1BB on total OT-II T cells was assessed after 3 d (*right*). All data are representative of three independent experiments. *$p < 0.05$.

Discussion

A growing number of autoinflammatory and allergic disorders have been identified in humans, most of which are attributed to hyperresponsiveness of T lymphocytes against potentially harmless foreign or self-antigens. T cells have multiple ways to limit their responsiveness to nonpathogenic stimuli. In the present study, we demonstrate that ligation of 4-1BBL on T cells can function in this regard and suppress T cell activation and early expansion of effector T cells. 4-1BBL was induced upon Ag-dependent activation of T cells under tolerizing conditions in vitro as well as in vivo and was active in this inhibitory capacity when inflammation and Ag presentation were suboptimal.

Although much of the literature on the interaction of 4-1BB and 4-1BBL has focused on the stimulatory capacity of 4-1BB on various cell types, including T cells, there is a growing body of research showing that 4-1BBL itself can signal. However, whether this provides a stimulatory or inhibitory stimulus appears to vary and may be both cell type specific and context-dependent. 4-1BBL signaling has been illustrated in myeloid cells as well as in lymphocytes. Cross-linking of 4-1BBL with immobilized 4-1BB–Fc, anti–4-1BBL mAb, or 4-1BB–expressing cells can lead to monocyte and DC activation, proliferation, maturation, production of proinflammatory cytokines such as IL-6, IL-8, TNF, or IL-12, and/or cell survival (11, 17–22). However, there is accumulating evidence of negative regulatory roles for 4-1BBL signaling in activation and/or differentiation of varying types of cells, including bone marrow myeloid precursors, osteoclasts, as

well as T cells (3, 4, 9, 10, 12, 23, 24). In particular, Schwarz et al. (12) first suggested the potential regulatory activity of 4-1BBL signaling by showing suppressed proliferation of human PBMC T cells when cocultured with fixed 4-1BB–expressing cells. The inhibitory activity of 4-1BBL signaling was not restricted to human PBMCs, but was also replicated in mouse splenocytes stimulated with anti-CD3 where the proliferation of 4-1BB–deficient T cells was reduced again when these cells were incubated with 4-1BB–expressing cells (3, 25).

We now add to these studies and suggest the suppressive activity of 4-1BBL on T cells is regulated by its own receptor. 4-1BB has long been known as an inducible molecule on T cells that transmits costimulatory signals to augment division, survival, and cytokine production under inflammatory conditions. We found that when 4-1BB was strongly induced, this resulted in the downregulation of 4-1BBL via a *cis* interaction that may largely occur on the T cell surface but might also be functional intracellularly. This was most dramatically illustrated with T cells that could not express 4-1BB where 4-1BBL was readily and easily detected at high levels on the cell surface. The plausible mechanisms limiting expression of 4-1BBL included shedding or cleavage from the membrane as well as internalization. We do not favor the former because although we could detect soluble 4-1BBL in the supernatant of activated T cells, we found as much, and in some cases more, soluble 4-1BBL in cultures of 4-1BB$^{-/-}$ T cells (data not shown). This is opposite to what would be predicted if a *cis* interaction with 4-1BB enhanced shedding or cleavage. In contrast, our data with confocal analyses and using endocytosis inhibitors support the idea that 4-1BB binding to 4-1BBL results in its internalization and subsequent degradation. This appears to be a mechanism by which the strong induction of 4-1BB maximizes its potential to be a costimulatory molecule for the T cell when 4-1BBL is upregulated on APCs under inflammatory conditions, reducing any potential negative effect of its ligand when a T cell encounters an APC that expresses 4-1BB.

The physiological significance of the immunoregulatory role of 4-1BBL engagement on T cells will be primarily evident in noninflammatory states or tolerizing conditions based on our data in vitro and in vivo. The mechanism we describe depends on a source of 4-1BB in *trans*, and this is most likely provided by APCs that can express 4-1BB. These are largely predicted to be DCs. Given the inhibitory activity of 4-1BBL, it would make sense that 4-1BB was provided on a tolerogenic or regulatory DC. The most obvious example of this cell is the CD103$^+$ DCs found in the MLNs, which we previously described expressed 4-1BB directly ex vivo (5). In line with this, we showed that expression of 4-1BB on a subpopulation of MLN DCs coincided with those cells that had the greatest ability to make retinoic acid. Indeed, ligation of 4-1BB sustained the activity of the enzyme RALDH, which promotes retinoic acid production in these MLN DCs. 4-1BB signaling additionally promoted RALDH activity in splenic DCs when they upregulated 4-1BB after stimulation with TLR2 ligand or GM-CSF. This in turn led to a greater ability of the DCs to promote the generation of Foxp3$^+$ Treg cells (5). Therefore, by blocking T cell activation through 4-1BBL, as well as upregulating retinoic acid production through 4-1BB signaling to the DCs, there can be synergistic suppression of the T cell response. As we show in vitro, this may in some cases also aid the generation of Foxp3$^+$ Treg cells. Such bidirectional signaling activity is likely to occur in mucosal tissues such as the gastrointestinal tract where TGF-β is available along with constant or periodic low-level stimulation of T cells by innocuous Ags from food or commensals. This hypothesis is further supported by our unpublished data, which show reduced numbers of Foxp3$^+$ Treg cells in the Peyer's patches and small intestinal lamina propria of 4-1BB$^{-/-}$ and 4-1BBL$^{-/-}$ mice.

In summary, we show a rate-limiting activity of 4-1BBL when expressed by recently activated T cells that suppresses T cell activation and effector cell development under tolerizing conditions or conditions where Ag presentation occurs at a low level with little inflammation. The data highlight the complex interplay between ligands and receptors in the TNF/TNFR superfamily. How intracellular signals from 4-1BBL exert a suppressive effect in T cells is not known, but understanding this will be important for future studies.

Disclosures

The authors have no financial conflicts of interest.

References

1. Croft, M. 2009. The role of TNF superfamily members in T-cell function and diseases. *Nat. Rev. Immunol.* 9: 271–285.
2. Croft, M., W. Duan, H. Choi, S. Y. Eun, S. Madireddi, and A. Mehta. 2012. TNF superfamily in inflammatory disease: translating basic insights. *Trends Immunol.* 33: 144–152.
3. Kwon, B. S., J. C. Hurtado, Z. H. Lee, K. B. Kwack, S. K. Seo, B. K. Choi, B. H. Koller, G. Wolisi, H. E. Broxmeyer, and D. S. Vinay. 2002. Immune responses in 4-1BB (CD137)-deficient mice. *J. Immunol.* 168: 5483–5490.
4. Lee, S. W., Y. Park, T. So, B. S. Kwon, H. Cheroutre, R. S. Mittler, and M. Croft. 2008. Identification of regulatory functions for 4-1BB and 4-1BBL in myelopoiesis and the development of dendritic cells. *Nat. Immunol.* 9: 917–926.
5. Lee, S. W., Y. Park, S. Y. Eun, S. Madireddi, H. Cheroutre, and M. Croft. 2012. Cutting edge: 4-1BB controls regulatory activity in dendritic cells through promoting optimal expression of retinal dehydrogenase. *J. Immunol.* 189: 2697–2701.
6. Goodwin, R. G., W. S. Din, T. Davis-Smith, D. M. Anderson, S. D. Gimpel, T. A. Sato, C. R. Maliszewski, C. I. Brannan, N. G. Copeland, N. A. Jenkins, et al. 1993. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur. J. Immunol.* 23: 2631–2641.
7. Wang, C., G. H. Lin, A. J. McPherson, and T. H. Watts. 2009. Immune regulation by 4-1BB and 4-1BBL: complexities and challenges. *Immunol. Rev.* 229: 192–215.
8. Shao, Z., and H. Schwarz. 2011. CD137 ligand, a member of the tumor necrosis factor family, regulates immune responses via reverse signal transduction. *J. Leukoc. Biol.* 89: 21–29.
9. Saito, K., N. Ohara, H. Hotokezaka, S. Fukumoto, K. Yuasa, M. Naito, T. Fujiwara, and K. Nakayama. 2004. Infection-induced up-regulation of the costimulatory molecule 4-1BB in osteoblastic cells and its inhibitory effect on M-CSF/RANKL-induced in vitro osteoclastogenesis. *J. Biol. Chem.* 279: 13555–13563.
10. Shin, H. H., J. E. Lee, E. A. Lee, B. S. Kwon, and H. S. Choi. 2006. Enhanced osteoclastogenesis in 4-1BB-deficient mice caused by reduced interleukin-10. *J. Bone Miner. Res.* 21: 1907–1912.
11. Kang, Y. J., S. O. Kim, S. Shimada, M. Otsuka, A. Seit-Nebi, B. S. Kwon, T. H. Watts, and J. Han. 2007. Cell surface 4-1BBL mediates sequential signaling pathways "downstream" of TLR and is required for sustained TNF production in macrophages. *Nat. Immunol.* 8: 601–609.
12. Schwarz, H., F. J. Blanco, J. von Kempis, J. Valbracht, and M. Lotz. 1996. ILA, a member of the human nerve growth factor/tumor necrosis factor receptor family, regulates T-lymphocyte proliferation and survival. *Blood* 87: 2839–2845.
13. Michel, J., S. Pauly, J. Langstein, P. H. Krammer, and H. Schwarz. 1999. CD137-induced apoptosis is independent of CD95. *Immunology* 98: 42–46.
14. Kim, W., J. Kim, D. Jung, H. Kim, H. J. Choi, H. R. Cho, and B. Kwon. 2009. Induction of lethal graft-versus-host disease by anti-CD137 monoclonal antibody in mice prone to chronic graft-versus-host disease. *Biol. Blood Marrow Transplant.* 15: 306–314.
15. Madireddi, S., S. Y. Eun, S. W. Lee, I. Nemčovičová, A. K. Mehta, D. M. Zajonc, N. Nishi, T. Niki, M. Hirashima, and M. Croft. 2014. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J. Exp. Med.* 211: 1433–1448.
16. Kang, J., S. J. Huddleston, J. M. Fraser, and A. Khoruts. 2008. De novo induction of antigen-specific CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in vivo following systemic antigen administration accompanied by blockade of mTOR. *J. Leukoc. Biol.* 83: 1230–1239.
17. Langstein, J., J. Michel, J. Fritsche, M. Kreutz, R. Andreesen, and H. Schwarz. 1998. CD137 (ILA/4-1BB), a member of the TNF receptor family, induces monocyte activation via bidirectional signaling. *J. Immunol.* 160: 2488–2494.
18. Langstein, J., J. Michel, and H. Schwarz. 1999. CD137 induces proliferation and endomitosis in monocytes. *Blood* 94: 3161–3168.
19. Pauly, S., K. Broll, M. Wittmann, G. Giegerich, and H. Schwarz. 2002. CD137 is expressed by follicular dendritic cells and costimulates B lymphocyte activation in germinal centers. *J. Leukoc. Biol.* 72: 35–42.
20. Kim, Y. J., G. Li, and H. E. Broxmeyer. 2002. 4-1BB ligand stimulation enhances myeloid dendritic cell maturation from human umbilical cord blood CD34$^+$ progenitor cells. *J. Hematother. Stem Cell Res.* 11: 895–903.
21. Lippert, U., K. Zachmann, D. M. Ferrari, H. Schwarz, E. Brunner, A. H. Mahbub-Ul Latif, C. Neumann, and A. Soruri. 2008. CD137 ligand reverse

signaling has multiple functions in human dendritic cells during an adaptive immune response. *Eur. J. Immunol.* 38: 1024–1032.
22. Kim, D. K., S. C. Lee, and H. W. Lee. 2009. CD137 ligand-mediated reverse signals increase cell viability and cytokine expression in murine myeloid cells: involvement of mTOR/p70S6 kinase and Akt. *Eur. J. Immunol.* 39: 2617–2628.
23. Shin, H. H., E. A. Lee, S. J. Kim, B. S. Kwon, and H. S. Choi. 2006. A signal through 4-1BB ligand inhibits receptor for activation of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis by increasing interferon (IFN)-β production. *FEBS Lett.* 580: 1601–1606.
24. Senthilkumar, R., and H. W. Lee. 2009. CD137L- and RANKL-mediated reverse signals inhibit osteoclastogenesis and T lymphocyte proliferation. *Immunobiology* 214: 153–161.
25. Lee, S. W., A. T. Vella, B. S. Kwon, and M. Croft. 2005. Enhanced CD4 T cell responsiveness in the absence of 4-1BB. *J. Immunol.* 174: 6803–6808.