In Vivo 4-1BB Deficiency in Myeloid Cells Enhances Peripheral T Cell Proliferation by Increasing IL-15

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In Vivo 4-1BB Deficiency in Myeloid Cells Enhances Peripheral T Cell Proliferation by Increasing IL-15


4-1BB signals are considered positive regulators of T cell responses against viruses and tumors, but recent studies suggest that they have more complex roles in modulating T cell responses. Although dual roles of 4-1BB signaling in T cell responses have been suggested, the underlying mechanisms are still not fully understood. In this study, we tested whether 4-1BB expression affected T cell responses differently when expressed in myeloid versus lymphoid cells in vivo. By assessing the proliferation of 4-1BB+/+ and 4-1BB−/− T cells in lymphocyte-deficient RAG2−/− and RAG2−/−/4-1BB−/− mice, we were able to compare the effects on T cell responses of 4-1BB expression on myeloid versus T cells. Surprisingly, adoptively transferred T cells were more responsive in tumor-bearing RAG2−/−/4-1BB−/− mice than in RAG2−/− mice, and this enhanced T cell proliferation was further enhanced if the T cells were 4-1BB deficient. Dendritic cells (DCs) rather than NK or tissue cells were the myeloid lineage cells primarily responsible for the enhanced T cell proliferation. However, individual 4-1BB−/− DCs were less effective in T cell priming in vivo than 4-1BB+/+ DCs; instead, more DCs in the secondary lymphoid organs of RAG2−/−/4-1BB−/− mice appeared to induce the enhanced T cell proliferation by producing and transpresenting more IL-15. Therefore, we conclude that in vivo 4-1BB signaling of myeloid cells negatively regulates peripheral T cell responses by limiting the differentiation of DCs and their accumulation in secondary lymphoid organs. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: BM, bone marrow; CBA, cytometric bead array; CD62L, L-selectin; CD11c, dendritic cell; InL, inguinal lymph node; LCMV, lymphocytic choriomeningitis virus; Th1, T helper type 1; Th2, T helper type 2; WT, wild-type.

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Materials and Methods

Mice, reagents, and Abs

Homzygous 4-1BB-/− B6 mice were crossed with RAG2-/− B6 mice (Taconic Farms) to generate RAG2-/−/− 4-1BB-/−/− mice. C57BL/6 mice were purchased from Charles River Laboratories (Orient Bio, Sungnam, South Korea). Pmel-1 TCR transgenic mice that recognize an H-2d-restricted epitope of gp100 (KPVPRNQDWL) and OT-I TCR transgenic C57BL/6 mice expressing an H-2k-restricted TCR specific for the OVA epitope (SIINFEKL) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific-pathogen-free conditions in the animal facility of the National Cancer Center in Korea. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the National Cancer Center and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Antibodies for intracellular staining (PK136) hybridoma cells were gifts from the American Type Culture Collection. All Abs for flow cytometry were purchased from eBioscience (San Diego, CA). Mouse CD4 and CD8 microbeads were from Miltenyi Biotec (Auburn, CA). Functional grade anti-mouse IL-15R/IL-15 complex mAb, mouse IL-15/IL-15R complex recombinant protein, and mouse IL-15R-IL-15 ELISA kits were from eBioscience. Clodronate (ClMDP) was a gift from Roche Diagnostics (Mannheim, Germany). The BD CBA Mouse Inflammation Kit was from BD Biosciences (San Jose, CA), and LPS was from List Biological Laboratories (Campbell, CA).

Tumor challenge and treatment

CD8 T and CD4 T cells were isolated from lymph nodes (LNs) and spleens of wild-type (WT) and 4-1BB-/−/− C57BL/6 mice using CD8 microbeads (Miltenyi Biotec). Purified 4-1BB+/+ and 4-1BB−/− CD4+ or CD8+ T cells were injected i.v. into RAG2-/− and RAG2-/−/− 4-1BB−/−/− mice at doses of 1 × 10⁶ cells/mouse. The mice were further challenged s.c. with 5 × 10⁶ MC38 adenocarcinoma cells. To deplete NK cells, mice receiving CD8+ T cells and MC38 tumor cells were further administered i.p. with PK136 anti-NK1.1 mAb on days 0 and 5. To deplete phagocytic cells, tumor-challenged mice were administered i.p. with 200 µl ClMDP liposomes or control liposomes on days 2, 4, and 6. To neutralize IL-15/IL-15Rα, mice were injected i.v. with anti-mouse IL-15/IL-15 complex complex mAb (2 µg/g body weight; eBioscience) 0, 5, and 10 d after MC38 tumor challenge.

Flow cytometry

To analyze the transferred CD8+ or CD4+ T cells, lymphocytes from LNs and spleens were preincubated with Fc blocker 24G2, stained with anti-CD11c-PE or anti-CD44-PE along with anti-CD8-PE-Cy5 or anti-CD4+ T cells. On day 15, spleen and InLN cells were prepared from each group of mice. The cells were counted and stained with anti-CD8-PE and anti-CD4+ T cells. Serum was collected from each mouse. Serum cytokines were measured by flow cytometry, and the percentages of CD4+ T, CD8+ T, and NK cells in the samples were measured by flow cytometry, and the absolute numbers of each cell type were calculated by multiplying the total number of viable cells by the calculated percentages.

Serum cytokines

Fourteen days after RAG2+/+ and RAG2−/− 4-1BB−/− mice had been challenged with MC38 tumor cells and had received CD4+ T or CD8+ T cells, serum was collected from each mouse. Serum cytokines were quantified using a cytometric bead array (CBA) kit (BD Biosciences) on a FACSCalibur cytometer equipped with CellQuestPro and CBA software.

Generation of BM chimeric mice

To generate BM chimeras, BM cells were isolated from the femurs and tibias of WT and 4-1BB−/− B6 mice and injected into irradiated (1200 rad) 5-wk-old WT or 4-1BB−/− B6 mice. After injection, the recipient mice were placed on acidified, antibiotic water and maintained on this water for 2 wk. To measure the reconstitution rate of the transferred cells, some of the mice were given BM cells from Thy1.1 congenic mice. Six weeks later, chimerism was assessed in the blood of the mice reconstituted with Thy1.1 BM cells (data not shown). The BM-reconstituted B6 mice were challenged s.c. with 5 × 10⁶ MC38 cells, and tumor growth was monitored daily.

In vivo CD8+ T cell priming with OVA-pulsed DCs

CD11c+ DCs were isolated from 4-1BB+/+ and 4-1BB−/− B6 mice and cultured in high glucose DMEM supplemented with 10% FBS in the presence of 100 µg OVA protein for 12 h. CD8+ T cells were isolated from OVA-specific OT-I mice using CD8-microbeads (Miltenyi Biotec) and labeled with 10 µm CFSE. RAG2−/−/− mice were first injected i.v. with CFSE-labeled OT-I CD8+ T cells (2 × 10⁶ cells/mouse), and then, the OVA-pulsed DCs were injected into their footpads (2 × 10⁵ cells/footpad). Popliteal LNs (PLNs) were isolated from the mice on day 4, and the lymphocytes were stained with anti-CD8-PE-Cy5 mAb.

LPS-induced apoptosis of DCs

To induce apoptosis of DCs in vivo, RAG2−/− and RAG2−/− 4-1BB−/− mice were injected i.v. with 10 µg LPS and subsequently administered s.c. with 5 × 10⁶ MC38 tumor cells. CD8+ T cells from WT B6 mice were injected i.v. into the mice 1, 4, and 7 d after the administration of LPS and MC38 tumor cells. On day 21, PLN cells from each group of mice were counted, stained with anti-CD44-FITC and anti–CD8-PE, and analyzed by FACS caliber (BD Biosciences).

Homeostatic proliferation of CD8+ T cells

CD8 T cells were isolated from LNs and spleens of C57BL/6 mice. Their naive status (CD62Lhigh/CD44low) was confirmed by staining the purified CD8+ T cells for L-selectin (CD62L) and CD44, and ~85% of the T cells were of the naive phenotype. The purified CD8+ T cells were labeled with 10 µC CFSE and 2 × 10⁵ T cells were injected i.p. per mouse into RAG2+/+ and RAG2−/− 4-1BB−/− mice. After 7 d, single-cell suspensions of inguinal draining lymph nodes (InLNs) were stained with anti–CD8-PE and CFSE, and dilutions of the transferred CD8+ T cells were analyzed by flow cytometry.

RT-PCR and ELISA of IL-15

RAG2−/− and RAG2−/− 4-1BB−/− mice were administered s.c. with 5 × 10⁶ MC38 tumor cells. Serum, InLNs and spleens were collected from each group of mice on day 14. Total RNAs were extracted from the homogenized InLNs and from CD11c+ DCs, which were isolated using CD11c microbeads from the collagenase-treated LN cells, and reverse transcribed into cDNA using a SuperScriptIII (Invitrogen Life Technologies). Transcript levels of IL-15 were assessed using the IL-15–specific primer set (forward, 5′-CATATGGGACCTCAACTGGAATGATGTAAGA-TA-3′; reverse, 5′-CATATGCTGAGGAGGTGTGATTGAACAT-3′). For IL-15/IL-15Rα ELISA, LNs and spleens were collected from RAG2−/− and RAG2−/− 4-1BB−/− mice 14 d after MC38 tumor challenge, weighed, and suspended in PBS at 500 mg/ml. The samples were homogenized and centrifuged to remove tissue debris. Concentrations of IL-15/IL-15Rα complex were assessed in serum and lysates of LNs and spleens using a Mouse IL-15/IL-15R Complex ELISA kit (eBioscience), according to the manufacturer’s instructions.

Adoptive transfer of naive and activated Pmel-1–specific CD8+ T cells

Naive pml-1 CD8+ T cells were prepared by freshly isolating CD8+ T cells from LNs and spleen of pml-1 transgenic B6 mice. Activated pml-1 CD8+ T cells were prepared by culturing the isolated pml-1 CD8+ T cells with 5 µg/ml hgp100 AA25-33 for 4 d, RAG2−/− and RAG2−/− 4-1BB−/− mice were injected s.c. with 2 × 10⁶ B16-F10 melanoma cells and further administered i.v. with 1 × 10⁶ naive or activated pml-1 CD8+ T cells. On day 15, spleen and InLN cells were prepared from each group of mice. The cells were counted and stained with anti-CD8-PE and anti-CD62L mAb, respectively.

Statistical analysis

Data were analyzed with the statistical program (Prism 4.0; GraphPad, San Diego, CA). Student t test was used to determine the statistical significance of differences between groups.

Results

Enhanced proliferation of CD8+ T cells in RAG2−/− 4-1BB−/− mice but not in RAG2−/−/− mice

To determine whether T cell responses were affected by 4-1BB deficiency of the myeloid cells, CD8+ T cells from WT or 4-1BB−/− mice...
B6 mice were adoptively transferred into 4-1BB−/− and 4-1BB−/− mice in a C57BL/6 background and challenged s.c. with MC38 adenocarcinoma cells. Tumor growth 14 d after tumor challenge was lower in the RAG2−/− 4-1BB−/− mice than in the RAG2−/− mice, independent of 4-1BB expression by the CD8+ T cells (Fig. 1A).

In addition, on day 14, both 4-1BB+/+ and 4-1BB−/− CD8+ T cells were more numerous in the InLNs and spleens of the RAG2−/− 4-1BB−/− mice than of the RAG2−/− mice (Fig. 1B). Moreover, there were more CD8+ T cells when 4-1BB−/CD8+ T cells were introduced into the RAG2−/− 4-1BB−/− mice than when 4-1BB+/+ CD8+ T cells were introduced (Fig. 1B). The absolute numbers of total spleen and InLN cells were ~2-fold higher in the RAG2−/− 4-1BB−/− mice than in the RAG2−/− mice whether the CD8+ T cells expressed 4-1BB (Fig. 1C, left). CD8+ T cell numbers in the spleen were 0.42 ± 0.023 × 10^6 and 0.32 ± 0.011 × 10^6 cells in the RAG2−/− mice receiving 4-1BB+/+ and 4-1BB−/− CD8+ T cells, respectively, and 1.58 ± 0.14 × 10^6 and 4.49 ± 0.48 × 10^6 cells in the RAG2−/− 4-1BB−/− mice receiving 4-1BB+/+ and 4-1BB−/− CD8+ T cells, respectively (Fig. 1C, upper middle). Thus, the CD8+ T cells significantly increased in the RAG2−/− 4-1BB−/− mice than in the RAG2−/− mice. Significant increases of absolute numbers of total and CD8+ T cells were also found in the InLNs (Fig. 1C, lower panels). However, NK cell numbers were comparable in the two groups (Fig. 1C, right).

There were no significant changes in the serum cytokine levels of IL-6, IL-10, MCP-1, and IL-12 in either group of mice on day 14. However, IFN-γ and TNF-α increased in the RAG2−/− 4-1BB−/− mice following transfer of 4-1BB+/+CD8+ T cells but not of 4-1BB−/CD8+ T cells (Fig. 1D); this indicates that 4-1BB expression in the CD8+ T cells is crucial for the increase of IFN-γ and TNF-α in the sera of RAG2−/− 4-1BB−/− mice. This finding indicates that 4-1BB deficiency of non-T cells was essential for the induction of enhanced CD8+ T cell proliferation and that the CD8+ T cell proliferation was further enhanced by 4-1BB deficiency of the CD8+ T cells.

**Proliferation of CD4+ T cells are enhanced in RAG2−/− 4-1BB−/− mice but not in RAG2−/− mice**

We next examined whether CD4+ T cell proliferations were also enhanced in the RAG2−/− 4-1BB−/− mice. When the above experiment was repeated with CD4+ T cells, tumor growth was similar in the experimental groups on day 14 (Fig. 2A). Nevertheless, LNs and spleens were larger on day 14 in the RAG2−/− 4-1BB−/− mice than in the RAG2−/− mice (data not shown). The proportions of the transferred CD4+ T cells in the spleen were ~5–7% in the RAG2−/− mice and ~9–10% in the RAG2−/− 4-1BB−/− mice (Fig. 2B, upper panels). NK cell frequencies were comparable in the two groups (Fig. 2B, lower panels). When we determined the absolute numbers of CD4+ T cells and NK cells in LNs and spleens, both cell types were much more numerous in the RAG2−/− 4-1BB−/− mice than the RAG2−/− mice because the total numbers of lymphocytes were substantially higher in the RAG2−/− 4-1BB−/− mice (Fig. 2C). The increases in total, CD4+ T, and NK cells were more evident when the RAG2−/− 4-1BB−/− mice received 4-1BB−/− rather than 4-1BB−/− B6 mice. 

**FIGURE 1.** CD8+ T cell responses against tumors in RAG2−/− and RAG2−/− 4-1BB−/− mice. CD8+ T cells from WT (4-1BB+/+) or 4-1BB−/− B6 mice were injected i.v. into RAG2−/− and RAG2−/− 4-1BB−/− mice at 1 × 10^6 cells/mouse, and 5 × 10^5 of MC38 adenocarcinoma cells were subsequently injected s.c. on the backs of the mice. (A) Tumor volumes 14 d after the tumor challenge. (B) Lymphocytes from spleens and InLNs on day 14 were stained with anti–CD11c-PE or anti–CD44-PE along with anti–CD8-PE-Cy5 and analyzed by FACSCalibur (BD Biosciences). (C) Absolute numbers of total lymphocytes, CD8+ T cells, and NK cells in spleens and InLNs. (D) Sera were collected from each group of mice on day 14, and serum cytokines were assessed using a BD CBA Mouse Inflammation kit. Data are representative of three independent experiments. Results in (C) and (D) are mean ± SD (n = 4 mice/group; *p < 0.05, **p < 0.001).
with 1 × 10^5 4-1BB+/+ or 4-1BB−/− CD4+ T cells and subsequently administered s.c. with 5 × 10^5 MC38 tumor cells. (A) Tumor volumes on day 14. (B) Splenocytes were first incubated with Fc blocker and stained with anti–CD4-PE and anti–CD44-FITC, or anti–CD27-PE and anti–NK1.1-FITC, for flow cytometry. (C) Absolute numbers of total lymphocytes, CD4+ T cells, and NK cells in spleens and InLNs. (D) Serum cytokines were measured using a BD CBA Mouse Inflammation kit on day 14. Data are representative of three independent experiments. Results in (C) and (D) are mean ± SD (n = 4 mice/group; *p < 0.05, **p < 0.01, ***p < 0.001).

T cells (Fig. 2C). There were no significant changes in serum levels of the inflammatory cytokines IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12 (Fig. 2D).

These results indicate that CD4+ T cell proliferations were also enhanced in RAG2−/−/4-1BB−/− mice compared with that of RAG2−/− mice, which again demonstrates that the enhanced proliferation of T cells was induced by 4-1BB deficiency of non-T cells, and further strengthened by 4-1BB deficiency of the T cells.

Tissue cells do not need to express 4-1BB to induce the enhanced T cell proliferation

We previously reported that stimulation of 4-1BB had a strong anticancer effect, and 4-1BB−/− B6 mice were also remarkably resistant to tumor growth (16). The therapeutic effects of 4-1BB triggering were dependent mainly on CD8+ T cells and partly on NK cells, whereas CD8+ T cells and NK cells were equally needed for suppressing tumor growth in 4-1BB−/− mice (16). In agreement with these results, tumor cells grew equally well in lymphocyte-deficient RAG2−/− and RAG2−/−/4-1BB−/− mice (16). However, because 4-1BB is expressed in lymphoid and nonlymphoid cells (18), we could not exclude the possibility that 4-1BB–deficient nonlymphoid cells also contributed to the enhanced T cell proliferation. Therefore, we generated BM chimeric mice by reconstituting WT and 4-1BB−/− mice with 4-1BB–intact or 4-1BB–deficient BM cells (Fig. 3A). The chimeric mice were challenged s.c. with MC38 tumor cells 6 wk after BM reconstitution. Tumor growth was normal in the mice reconstituted with 4-1BB+/+ BM cells but inhibited in the mice that received 4-1BB−/− BM cells, independent of 4-1BB expression in the recipient mice (Fig. 3B). Flow cytometric analysis on day 14 indicated that CD4+ T and CD8+ T cells had increased in the InLNs of the mice that received 4-1BB−/− BM cells but not in their spleens (Fig. 3C). H&E staining of tumor tissue showed that there were more necrotic tumor cells and tumor-infiltrating immune cells in the 4-1BB−/− BM-reconstituted mice than in the 4-1BB+/+ BM-reconstituted mice (Fig. 3D).

Because the MC38 tumor cells did not express 4-1BB and 4-1BBL on their surface (data not shown), these results clearly demonstrate that the enhanced T cell proliferation in 4-1BB−/− mice was exclusively induced by immune cells rather than 4-1BB–expressing tumor or tissue cells.

NK cells are not essential for the enhanced T cell proliferation of RAG2−/−/4-1BB−/− mice

We previously reported that NK cells are involved in the enhanced antitumor responses of 4-1BB−/− mice by helping antitumor CD8+ T cells rather than by directly killing tumor cells (16). On the basis of these previous results, we next asked whether the enhanced T cell proliferation of 4-1BB−/−/4-1BB−/− mice would be reversed by depleting their NK cells. RAG2−/− and RAG2−/−/4-1BB−/− mice were injected i.v. with CD8+ T cells and challenged with MC38 tumor cells as described previously. NK cells were then depleted by injecting the mice with PK136 anti-NK1.1 mAb on days 0, 5, and 10. Flow cytometric analysis on day 12 confirmed that NK cells had been completely eliminated, whereas the proportions of CD8+ T cells had not been reduced (Fig. 4A). The absolute numbers of total and CD8+ T cell in the RAG2−/−/4-1BB−/− mice were also not...
reduced by NK cell depletion but rather increased (Fig. 4B). These results show that NK cells are not essential for the induction of enhanced T cell proliferation.

APCs induce the enhanced T cell proliferation in RAG2−/− 4-1BB−/− mice in vivo

We next suspected that APCs such as monocytes/macrophages and DCs might be the cause of the enhanced T cell proliferation in RAG2−/− 4-1BB−/− mice. We therefore depleted partially the APCs by injecting Cl2MDP liposomes into RAG2−/− and RAG2−/− 4-1BB−/− mice that had received CD8+ T cells and MC38 tumor cells, as described above. When we examined the mice on day 12, we found that the injection of Cl2MDP liposomes had prevented the enhanced T cell proliferation of the CD8+ T cells in RAG2−/− 4-1BB−/− mice by significantly decreasing total and CD8+ T cell numbers in InLNs (Fig. 5A).

FIGURE 3. CD8+ T responses in bone marrow-reconstituted 4-1BB+/+ and 4-1BB−/− B6 mice. (A) 4-1BB+/+ and 4-1BB−/− B6 mice received a lethal dose of gamma irradiation (1200 rad whole-body irradiation). Four hours later, they were reconstituted with 1 × 107 T cell–depleted BM cells from WT or 4-1BB−/− B6 mice and maintained for 6 wk. (B) Four different BM chimeric mice were injected s.c. with 2 × 106 MC38 adenocarcinoma cells on the back, and tumor growth was monitored on the indicated days. (C) Spleen and InLN cells were isolated on day 14 and stained with anti–CD4-FITC and anti–CD8–PE. All samples were subsequently analyzed by FACS Calibur. (D) Tumor tissues were paraffin-embedded on day 20, and sections were stained with H&E solution (original magnification ×40). Data are representative of two independent experiments. Results in (C) are mean ± SD (n = 4 mice/group; *p < 0.05, **p < 0.01).

FIGURE 4. Enhanced T cell proliferation of 4-1BB−/− mice in the absence of NK cells. RAG2−/− and RAG2−/− 4-1BB−/− mice were injected i.v. with 1 × 106 of CD8+ T cells and subsequently administered s.c. with 5 × 106 MC38 tumor cells. To deplete NK cells, the mice were further given i.p. PK136 anti-NK1.1 mAb on days 0, 5, and 10. (A) Spleen and inguinal LN cells were stained with anti–CD11c–PE-Cy5, anti–NK1.1–PE, and anti–CD8–FITC 12 d after tumor challenge. All samples were subsequently analyzed by FACS Calibur. (B) Absolute numbers of total, CD8+ T cells, and NK cells in spleens and InLNs on day 10. Data are representative of two independent experiments. Results in (B) are mean ± SD (n = 3 mice/group; **p < 0.01, ***p < 0.001).
However, a previous report had indicated that adoptively transferred Ag-pulsed 4-1BB−/− DCs were less efficient at priming CD4+ T cells in vivo than 4-1BB+/+ DCs (19). Therefore, we wondered whether the adoptively transferred Ag-pulsed 4-1BB−/− DCs might lead to enhanced proliferation of CD8+ T cells in vivo despite not inducing CD4+ T cell proliferation efficiently. CD11c+ DCs were isolated from WT and 4-1BB−/− mice, pulsed with OVA for 12 h, and injected into the footpads of WT and 4-1BB−/− B6 mice that received CFSE-labeled OT-I CD8+ T cells. Again, 4-1BB deficiency of the DCs resulted in poor CD8+ T cell proliferation in RAG2−/− 4-1BB−/− mice, this is not due to an enhanced T cell priming ability of the individual APCs.

It has been shown that the 4-1BB/4-1BBL interaction negatively regulates myelopoiesis so that DC proportions are elevated in the secondary lymphoid organs of 4-1BB−/− and 4-1BBL−/− mice (17). Although 4-1BB−/− DCs did not prime T cells efficiently in vivo, the enhanced T cell proliferation in the RAG2−/−4-1BB−/− mice might have resulted from the accumulation and multiplication of DCs in secondary lymphoid organs. To test this possibility, we induced apoptosis of DCs in vivo by injecting LPS i.v. into MC38-bearing RAG2−/− and RAG2−/−4-1BB−/− mice (20), followed by CD8+ T cells 1, 4, or 7 d after the LPS. Because the apoptotic DCs would be progressively replaced with newly differentiated DCs, we suspected that the enhanced T cell proliferation in the RAG2−/−4-1BB−/− mice would diminish with time. However, when we analyzed CD8+ T cells on day 21, which was 14 d after the final injection of LPS, the percentages of CD44hi CD8+ T cells in InLNs were comparable in the LPS-injected RAG2−/− and RAG2−/−4-1BB−/− mice (Fig. 5C), and the absolute numbers of total and CD8+ T cells also indicated that the transferred CD8+ T cell proliferation was not enhanced in the LPS-injected RAG2−/−4-1BB−/− mice (Fig. 5D).

Taken together, these results indicate that DCs are primarily responsible for the induction of enhanced T cell proliferation in RAG2−/−4-1BB−/− mice but that this is not due to the enhanced T cell priming ability of individual 4-1BB−/− DCs.

**Blockade of IL-15/IL-15Ra prevents the enhanced T cell proliferation in RAG2−/−4-1BB−/− mice**

Although we found that DCs mediated the enhanced T cell proliferation in the RAG2−/−4-1BB−/− mice, it was not clear how this took place. We found by chance, when we transferred CFSE-labeled CD8+ T cells into RAG2−/− and RAG2−/−4-1BB−/− mice without tumor challenge as a control, that the CD8+ T cells divided more rapidly in the RAG2−/−4-1BB−/− mice than in RAG2−/− mice (Fig. 6A). Repeated experiments confirmed that the homeostatic proliferation of CD8+ T cells was greater in the RAG2−/−4-1BB−/− mice than in the RAG2−/− mice. Naive T cells require contact with IL-7 and self-peptide/MHC ligand for

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**FIGURE 5.** Enhanced T cell proliferation of 4-1BB−/− mice in the absence of APCs. RAG2−/− and RAG2−/−4-1BB−/− mice were injected i.v. with 1 × 10^6 CD8+ T cells and subsequently given s.c. 5 × 10^5 MC38 tumor cells. (A and B) To deplete phagocytic cells, the mice were further administered i.v. 200 μl i.C MDP–liposomes or liposome alone on days 4, 6, and 8. (A) Lymphocytes from spleens and InLNs were prepared 12 d after tumor challenge, counted, and stained with anti–CD8-FITC, anti–NK1.1-PE, and anti–CD11c-PE-Cy5. Absolute numbers of total, CD8+ T cells, and NK cells were calculated. (B) 4-1BB−/− and 4-1BB−/− mice were injected i.v. with CFSE-labeled OT-I CD8+ T cells and then administered OVA-pulsed DCs into the footpads, as described in Materials and Methods. On day 4, CFSE dilutions of the OT-I CD8+ T cells were measured in the PLNs. All samples were subsequently analyzed by FACS Calibur (BD Biosciences). Dividing cells (percentage) = (dividing cells percentage/dividing cell percentage + nondividing cell percentage) × 100. (C and D) RAG2−/− and RAG2−/−4-1BB−/− mice were injected i.v. with 10 μg LPS and s.c. with 5 × 10^5 MC38 tumor cells and further administered CD8+ T cells on days 1, 4, or 7, as described in Materials and Methods. (C) Flow cytometric analysis of CD44 expression by the CD8+ T cells in InLNs on day 21. (D and E) Absolute numbers of total and CD44hi CD8+ T cells in the InLNs were calculated. Data are representative of three independent experiments. Results in (A), (B), (D), and (E) are mean ± SD (n = 3 mice/group; *p < 0.05, **p < 0.01).
homeostatic survival and proliferation, whereas central memory and memory phenotype CD8+ T cells require IL-15 (21–23). Moreover, IL-7 is mainly produced by fibroblastic reticular cells in secondary lymphoid organs (24), whereas IL-15 is generated by APCs such as monocytes and DCs (25, 26). Because the enhanced T cell proliferation was mediated by APCs but not by tissue cells (Figs. 3B, 5A, 5C), we suspected that IL-15 might be implicated in this event. Indeed, when we measured IL-15/IL-15Rα levels in serum and lysates of the InLNs and spleens using a specific ELISA kit (eBioscience), IL-15 transcript levels were significantly elevated in lysates of the InLNs (Fig. 6B).

When IL-15 transcript levels were assessed in the total mRNA of whole InLNs and CD11c+ DCs 14 d after tumor injection, IL-15 transcript levels were higher in the whole InLNs of the RAG2−/− mice than in those of the RAG2−/− mice but similar in the CD11c+ DCs (Fig. 6C). Because previous reports indicated that numbers of DCs are elevated in 4-1BB−/− mice and also in RAG2−/− mice in steady state (17, 19), we also examined the frequencies of CD11c+ DCs in InLNs of RAG2−/− and RAG2−/− mice 2 wk after MC38 challenge. CD11c+ DC frequencies continued to be higher in the RAG2−/− mice than the RAG2−/− mice (Fig. 6D), which suggests that IL-15/IL-15Rα levels were also higher.

To see whether IL-15 was indeed the mediator of the enhanced T cell proliferation in RAG2−/− mice, the mice were injected with CD8+ T cells and MC38 tumor cells as described above, and some were further injected i.v. with blocking anti–IL-15/IL-15Rα mAb on days 0, 5, and 10. Two weeks later, lymphocytes from spleen and InLNs were counted and stained with anti–CD8β and anti-NK1.1 mAb. Again, total and CD8+ T cell numbers in the spleen and InLNs were significantly higher in the control RAG2−/−/4-1BB−/− mice than the RAG2−/− mice (Fig. 6E). However, treatment with anti–IL-15/IL-15Rα mAb significantly reduced total and CD8+ T cell numbers in the spleens and InLNs of the RAG2−/−/4-1BB−/− mice, but only moderately decreased total and CD8+ T cells in the InLNs, although not in the spleens of the RAG2−/− mice (Fig. 6E).

Taken together, these results indicate that the enhanced T cell proliferation is the result of increased IL-15/IL-15Rα levels in tumor-bearing RAG2−/− mice and that the IL-15 overproduction is due to more DCs in the RAG2−/−/4-1BB−/− mice.

Addition of IL-15/IL-15Rα complex fails to restore the enhanced T cell proliferation in the absence of APCs

Because there are reports that transpresentation of IL-15 is a mechanism by which its immunological effects are enhanced (27, 28), we examined whether injection of IL-15/IL-15Rα complex induced the proliferation of CD8+ T cells in Cl2MDP liposome–injected mice. RAG2−/− and RAG2−/−/4-1BB−/− B6 mice that received MC38 tumor cells and CD8+ T cells from C57BL/6 mice on day 0 were injected i.v. with PBS or Cl2MDP liposomes on days 5 and 10, and this was followed from day 6 by daily i.p. injection of IL-15/IL-15Rα complex for 6 d. Flow cytometric analysis of splenocytes on day 15 demonstrated that CD11c+ DCs...
were still reduced in number in the CI-MDP liposome–injected mice independent of the IL-15/IL-15Rα injection (Fig. 7A, left panel). The CD8+ T cell ratios in spleen and InLNs were minimally altered following injection of the CI-MDP–liposomes and/or IL-15/IL-15Rα complex (Fig. 7A, middle and right panels). Most of the CD8+ T cells were CD62Llow in the spleen and only a half of them were CD62Llow in the InLNs (Fig. 7A, middle and right panels). Moreover, the injection of CI-MDP–liposomes showed a tendency to reduce CD62Llow effector T cells (Fig. 7A, right panel). The CI-MDP–liposomes had a minimal effect on the frequencies of CD8+ T cells in the spleen and InLNs but markedly decreased total and CD8+ T cells in both spleen and InLNs (Fig. 7B, 7C). Administration of IL-15/IL-15Rα complex failed to reverse the reduction of total and CD8+ T cells caused by the clodronate–liposomes (Fig. 7B, 7C). These results suggest that although the enhanced CD8+ T cell proliferation in RAG2−/− mice is mediated by IL-15, the increased IL-15 may be transpresented by the APCs.

Enhanced proliferation of pmel-1–specific CD8+ T cells in RAG2−/− mice

Because the transferred CD8+ T cells included only small numbers of tumor-reactive T cells, many of the activated CD8+ T cells found in the spleen seemed to be bystander-activated CD8+ T cells. Therefore, we next examined tumor-specific CD8+ T cell responses by transferring naive or activated pmel-1–specific CD8+ T cells into B16-F10 melanoma–challenged RAG2−/− mice and RAG2−/−/4-1BB−/− mice. When freshly isolated pmel-1 CD8+ T cells were transferred into the melanoma-injected mice, the percentages of CD8+ T cells in InLNs and spleen were again higher in the RAG2−/−/4-1BB−/− mice than the RAG2−/− mice, and half of them were CD62Llow effector CD8+ T cells (Fig. 8A). The absolute numbers of total and CD8+ T cells in the spleen and InLNs indicated that enhanced CD8+ T cell responses were only found in the InLNs of the RAG2−/−/4-1BB−/− mice not in the spleens (Fig. 8B). When pmel-1 CD8+ T cells that were activated with pmel-1 peptide for 4 d in vitro were adoptively transferred into melanoma-challenged mice, the frequencies of CD8+ T cells were higher in the InLNs and spleen of the RAG2−/−/4-1BB−/− mice than in those of the RAG2−/− mice, and most of them were CD62Llow effector CD8+ T cells (Fig. 8C). However, total numbers of cells in the spleen and InLNs were comparable in the RAG2−/−/4-1BB−/− and RAG2−/− mice, and so the total number of CD8+ T cells was moderately higher in the RAG2−/−/4-1BB−/− mice than the RAG2−/− mice (Fig. 8D).

These results indicate that proliferation of Ag-specific CD8+ T cells is enhanced in the tumor draining LNs of RAG2−/−/4-1BB−/− mice and that Ag presentation by APCs may be involved in the enhanced T cell proliferation of these mice.

**Discussion**

We have shown that 4-1BB in myeloid cells, particularly in APCs, negatively regulates peripheral T cell responses in vivo. Thus, adoptively transferred CD4+ and CD8+ T cells showed the enhanced T cell proliferation in tumor-bearing RAG2−/− mice, and DCs and IL-15 were primarily responsible for this enhanced T cell proliferation. However, isolated 4-1BB+/+ and 4-1BB−/− DCs made comparable levels of IL-15, and individual

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**FIGURE 7.** Minimal effects of IL-15/IL-15Rα complex on the CD8+ T cell proliferation in the absence of APCs. RAG2−/− and RAG2−/−/4-1BB−/− mice were injected i.p. with MC38 tumor cells and i.v. with CD8+ T cells from C57BL/6 mice as described above. PBS or clodronate liposome was injected i.p. to the tumor-bearing mice at days 5 and 10, and 1 µg IL-15/IL-15Rα complex (IL-15) was daily injected i.p. 6 times from day 6. Spleen and InLN cells were prepared from each group of mice 15 d after the tumor injection, and spleens were digested with collagenase and DNase I for 1 h. (A) To assess the frequency of APCs including DCs, splenocytes were stained with anti-CD11c and anti-CD11b mAb. Spleen and InLN cells were stained with anti-CD8β and anti-CD62L mAb. (B and C) Absolute numbers of total and CD8+ T cells in spleens and InLNs. Results in (B) and (C) are mean ± SD (n = 3 mice/group; *p < 0.05, **p < 0.01, ***p < 0.005).
4-1BB<sup>−/−</sup> DCs induced T cell responses in vivo less efficiently than 4-1BB<sup>+/+</sup> DCs. Therefore, we concluded that 4-1BB deficiency in myeloid cells in vivo induces the enhanced proliferation of peripheral T cells by promoting the accumulation of DCs in secondary lymphoid organs.

Although enhanced T cell responses were first reported when 4-1BB<sup>−/−</sup> mice were generated more than a decade ago (29), the underlying mechanism was not understood. Other studies have also pointed to a negative regulatory function of 4-1BB in modulating T cell responses. However, autoimmune diseases resembling human systemic lupus erythematosus and Sjögren syndrome developed more rapidly and were more frequent in 4-1BB–deficient mice than in 4-1BB<sup>+/+</sup> mice (34, 35). 4-1BB signaling in the myeloid cells rather than reverse signaling cause the T cell responses were enhanced in the absence of 4-1BB signals and were markedly increased in secondary lymphoid organs by enhancing their survival rate through overexpression of Bcl-2 or deletion of Bim or by injecting Flt3 ligand in vivo (36–38). T/B cell responses against specific Ags, or pathogens were markedly enhanced. These results indicate that 4-1BB<sup>−/−</sup> mice may gradually enhance myelopoiesis by continuously triggering TLR signaling and thus induce the enhanced T cell proliferation.

4-1BB is typically induced on activated peripheral T cells and NK cells, and these 4-1BB–expressing cells appear to migrate into the BM and trigger reverse signaling in progenitor cells through 4-1BB. Indeed, the enhanced T cell proliferation initiated by the 4-1BB deficiency of the T cells was further enhanced by 4-1BB deficiency of the myeloid cells. Also, there are reports that when DCs were increased in secondary lymphoid organs by enhancing their survival rate through overexpression of Bcl-2 or deletion of Bim or by injecting Flt3 ligand in vivo (36–38), T/B cell responses against specific Ags, or pathogens were markedly enhanced. These results indicate that 4-1BB<sup>−/−</sup> mice may gradually enhance myelopoiesis by continuously triggering TLR signaling and thus induce the enhanced T cell proliferation.
a negative impact on the development of CD8+ T cell immunity against LCMV by triggering perforin-mediated killing of T cells through activation of NKG2D on NK cells (41). It still remains unclear why NK cells showed different responses to the transferred CD4+ T and CD8+ T cells in RAG2−/−/4-1BB−/− mice (Figs. 1C, 2C). Because NK cells increased as CD4+ T cells were transferred in RAG2−/−/4-1BB−/− mice (Fig. 2C), IL-15 appeared to be involved in the expansion of NK cells along with CD4+ T cells. As for the CD8+ T cell transfer, a possible explanation could be that CD8+ T cells rapidly expanded in RAG2−/−/4-1BB−/− mice and ended up becoming a dominant population in lymphoid organs. Thus, the consequent limited space might have prevented NK cells from properly expanding even in the presence of IL-15. Alternatively, cytotoxic CD8+ T cells may suppress NK cell expansion as NK cells are known to do the same for CD8+ T cells (39). Indeed, in tumor-bearing mice that were treated with an agonistic anti–4-1BB mAb, the frequency and number of NK cells markedly decreased because of the increase of cytotoxic CD8+ T cells (16). Recent studies indicate that NK cells indirectly regulate T cell responses by lysing MCMV-infected APCs (40, 41) and have a negative impact on the development of CD8+ T cell immunity against LCMV by triggering perforin-mediated killing of T cells through activation of NKG2D on NK cells (39). These results implicate that the immune cells with cytotoxicity such as activated NK and CD8+ T cells could inhibit other types of immune cells. Taken together, although the increased IL-15 may have effects on NK cells in the initial phase of immune response, such effects of IL-15 may gradually diminish because of the increase of activated CD8+ T cells. Collectively, our data suggest that 4-1BB/4-1BBL interactions not only lead to enhanced proliferation and differentiation of T cells but also negatively regulate peripheral T cell responses, probably by limiting the maximum threshold of T cell responses in vivo.

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Disclosures

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