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Peripheral 4-1BB Signaling Negatively Regulates NK Cell Development through IFN-γ

Beom K. Choi,* † Young H. Kim, * † Chang H. Kim, * † Moon S. Kim, * † Kwang H. Kim, * † Ho S. Oh, * † Myoung J. Lee, * † Don K. Lee, * † Dass S. Vinay, ‡ and Byoung S. Kwon,* †

Stimulation of 4-1BB (CD137) was shown to produce strong anticancer effects in vivo. In contrast, 4-1BB−/− (4-1BB−/−) B6 mice are remarkably resistant to tumor growth. We set out to determine the mechanisms involved in these seemingly contradictory observations. We found that the therapeutic effects of 4-1BB triggering were mainly dependent on CD8+ T cells and partially on NK cells, whereas CD8+ T and NK cells were equally needed to suppress tumor growth in 4-1BB−/− mice. Cellular analysis showed that the frequency and number of NK cells in the spleen and bone marrow were decreased by 4-1BB triggering but were increased in the absence of 4-1BB signaling in tumor-challenged mice. The 4-1BB–mediated downregulation of NK cell development was primarily dependent on IFN-γ, which was produced by peripheral CD8+ T and NK cells. The suppression of NK cell development by 4-1BB–mediated IFN-γ production occurred in the bone marrow. As 4-1BB signaling increased in the periphery, more CD8+ T cells but fewer NK cells contributed to the antitumor immunity. As 4-1BB signaling decreased, more NK cells participated in the antitumor immunity. We conclude that 4-1BB signaling results in a shift of the dominant type of immune cell in antitumor immunity from the innate NK cell to the adaptive CD8+ T cell and that the level of IFN-γ is critical for this 4-1BB–mediated shift. The Journal of Immunology, 2010, 185: 1404–1411.

A n inducible costimulatory molecule, 4-1BB (CD137), is expressed on activated T cells, NK/NKT cells, CD4+ CD25+ regulatory T cells, and dendritic cells, and it positively regulates the functions of these immune cells by enhancing their proliferation and survival (1–5). Because of the several unique properties of 4-1BB triggering, including preferential expansion of CD8+ T cells, inhibition of activation-induced cell death, and selective induction of Th1-type cytokines, such as IFN-γ and TNF-α (6–8), agonistic anti–4-1BB mAb was shown to be an effective anticancer agent and is now in clinical trials (9).

Although 4-1BB signaling has profound effects on the activation and proliferation of T cells, 4-1BB–deficient (4-1BB−/−) mice do not show any defects in T cell development, but they have reduced NK and NKT cells and increased dendritic cells in secondary lymphoid organs (2, 10). Moreover, hyperproliferation of T cells occurs in 4-1BB−/− mice in response to mitogens (11, 12), but the mechanism remains to be elucidated. These data indicate that 4-1BB has more complex roles in the regulation and development of immune cells than simply regulating T cell-mediated immune responses (13).

We previously reported that 4-1BB−/− mice have reduced peripheral NK cells and, thus, showed enhanced tumor growth when the mice were challenged with NK cell-sensitive tumors, such as RMA-S that barely express MHC class I (2). In this article, we report that, in the case of CD8+ T cell-sensitive tumors, including EL4, MC38, CT26, and RENCA, 4-1BB−/− mice induce strong antitumor responses that suppress tumor growth. By comparing the antitumor responses in wild type (WT) and 4-1BB−/− mice with or without the treatment of agonistic anti-4-1BB mAb in vivo, we demonstrated that the antitumor response was mainly mediated by CD8+ T cells in the case of 4-1BB triggering but by CD8+ T and NK cells in the 4-1BB−/− mice. We also showed that strong 4-1BB signaling on peripheral T cells and NK cells suppressed NK cell development in the bone marrow (BM) by increasing IFN-γ levels. Because stronger 4-1BB signaling increased CD8+ T cell responses but gradually reduced NK cell responses in antitumor immunity, it seems that 4-1BB signaling regulates the balance between CD8+ T cell- and NK cell-mediated antitumor immunity by modulating IFN-γ levels. Our findings provide evidence that the intensity of peripheral immune responses modulates the development of innate and adaptive immune cells in the BM and that peripheral 4-1BB signaling contributes to the regulation of NK cell development.

Materials and Methods

Mice and reagents

Homozygous 4-1BB−/− B6 mice were crossed with RAG2−/− B6 mice (Taconic Farms, Germantown, NY) to generate 4-1BB−/−/RAG2−/− mice (double knockout [DKO]) (11). C57BL/6, IFN-γ−/−, and IFN-γR−/− C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions in the animal facility of the National Cancer Center in Korea and were used at 6–8 wk of age. 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. Agonistic anti-mouse 4-1BB mAb-producing

The online version of this article contains supplemental material.
hybridoma cells (3E1) were a gift from Dr. Robert Mittler (Emory University, Atlanta, GA), and anti–NK1.1 mAb-producing (PK136) and anti–CD8 mAb-producing (2.43) hybridoma cells were from the American Type Culture Collection (Manassas, VA). All Abs for flow cytometry were purchased from eBioscience (San Diego, CA). Recombinant adenoviruses expressing lacZ (Ad-lacZ) or IFN-γ (Ad–IFN-γ) were generous gifts from Dr. Sung-Young Chul (POSTECH, Pohang, Republic of Korea).

**Tumor challenge and treatment**

CT26 colon cancer cells and renal cancer cells (RENCA) were s.c. injected into 6- to 8-wk-old female BALB/c mice at doses of 3 × 10^6 and 2 × 10^6 cells/mouse, respectively. EL4 thymoma and MC38 adenocarcinoma cells were injected into C57BL/6 mice at doses of 2 × 10^6 and 2 × 10^5 cells, respectively. To hyperactivate 4-1BB signaling, tumor-challenged mice were treated i.p. with 100 μg agonistic anti–4-1BB mAb on postinjection (PI) days 5, 10, 15, and 20. To deplete NK or CD8+ T cells, tumor-challenged mice were injected i.p. with 400 μg anti-NK1.1 or anti–CD8 mAb. To increase serum IFN-γ levels in vivo, the tumor-challenged mice were injected i.v. with Ad–IFN-γ, or Ad-lacZ as a control, at 2 × 10^6 PFU per mouse on PI day 10. IFN-γ−/− (GKO) or IFN-γ−/− (GRKO) C57BL/6 mice were challenged s.c. with EL4 cells at doses of 2 × 10^6 cells. RAG2−/− or DKO B6 mice were injected s.c. with MC38 tumor cells at doses of 2 × 10^3 cells.

**Flow cytometry**

NK cells were detected by staining lymphocytes from spleen, lymph nodes (LNs), or BM with anti–NK1.1-PE and anti–CD3-PE-Cy5. To differentiate the stages of NK cell development, BM cells were stained with anti–NK1.1-PE, anti–CD94-PE, or anti–Ly49s-PE, as well as anti–CD122-FITC and anti–CD122-PE, CD122+CD3− cells were gated and analyzed for expression of NK1.1 (stage I/II), CD94 (stage II), and Ly49s (stage III), as previously reported (14). To count mature NK cells in the BM, the cells were stained with anti–NK1.1-PE, anti–CD3-PE-Cy5, and anti–CD11b-FITC. NK1.1+CD3− cells were gated and analyzed for expression of CD11b (stage V).

**Absolute numbers of NK cells**

Spleens, LNs, and BM cells were prepared from each group of mice, counted, and stained with fluorescein-conjugated Abs. The percentages of the NK subsets in the samples were measured by flow cytometry, and the absolute numbers of each type were calculated by multiplying the total number of viable cells by the calculated percentages.

**Serum cytokines**

Seven days after RAG2−/− or DKO B6 mice had been challenged with MC38 tumor cells, serum was collected from each mouse. Serum cytokines were quantified using a cytometric bead array kit (BD Biosciences, San Diego, CA) on a FACS Calibur cytometer equipped with CellQuestPro and CBA software.

**Statistical analysis**

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

**Results**

**Suppression of tumor growth by 4-1BB–proficient and 4-1BB–deficient mice**

To compare tumor growth in 4-1BB–proficient and 4-1BB–deficient mice we injected mice in a B6 background s.c. with MC38 colon adenocarcinoma or EL4 thymoma cells and injected them i.p. with agonistic anti–4-1BB mAb or rat IgG as a control. As reported previously (9), treatment with anti–4-1BB mAb induced a strong antitumor response and, thus, suppressed the growth of MC38 and EL4 cells (Fig. 1A).

When WT and 4-1BB−/− mice were challenged s.c. with tumor cells, such as MC38, EL4, CT26, or RENCA, which form primarily CD8+ T cell-sensitive tumors, surprisingly many of the 4-1BB−/− mice inhibited tumor growth more effectively than did the WT mice (Fig. 1B). This suggested that the antitumor response could be enhanced by 4-1BB triggering and by 4-1BB deficiency in vivo, which implied that 4-1BB signaling has complex roles in the regulation of immune responses.

**Essential requirement for NK cells to suppress tumor growth in 4-1BB−/− mice but not in mice receiving anti–4-1BB mAb**

Although it is well-known that strong 4-1BB triggering can eradicate tumors in various settings (15–17), suppression of tumor growth in 4-1BB−/− mice was not expected. To uncover the underlying mechanism of this enhanced antitumor response, we first examined the contributions of CD8+ T cells and NK cells, major immune cells that directly suppress tumor growth, in the therapeutic effects of 4-1BB triggering and deficiency.

EL4-challenged B6 mice were injected i.p. with anti–4-1BB mAb or rat IgG and further treated with NK- or CD8+ T cell-depleting mAb. Depletion of CD8+ T cells completely reversed the therapeutic effects of anti–4-1BB mAb; tumor volumes were comparable in rat IgG-treated and anti–4-1BB mAb-treated mice (Fig. 2A). In contrast, depletion of NK cells only partially reversed the therapeutic effects of anti-4-1BB mAb (Fig. 2B).

Depletion of CD8+ T cells again completely abolished the enhanced antitumor responses of 4-1BB−/− mice, and the tumor volumes of WT and 4-1BB−/− mice were comparable following removal of the CD8+ T cells (Fig. 2C). Depletion of NK cells totally reversed the therapeutic effects of 4-1BB−/− mice, much like CD8+ T cell depletion (Fig. 2D).

These results suggest that although WT mice receiving anti–4-1BB mAb and 4-1BB−/− mice showed similar enhanced antitumor responses, the underlying mechanisms were different, with NK cells contributing more to the suppression of tumor growth in the 4-1BB−/− mice than in B6 mice receiving anti–4-1BB mAb. CD8+ T cells were the major effectors after anti–4-1BB treatment, whereas cooperation between CD8+ Tand NK cells was required in the 4-1BB-deficient setting.

**Increase of NK cells in the periphery and BM in 4-1BB−/− mice**

Because NK cells were essential for suppressing tumor growth in 4-1BB−/− mice as opposed to WT B6 mice receiving anti–4-1BB
mAb, we examined the relationship between 4-1BB signaling and numbers of NK cells. We analyzed numbers of NK cells following anti–4-1BB treatment of EL4-challenged B6 mice. NK cells were barely detectable, but CD8+ T cells increased 3–4-fold in the spleens of these mice (Fig. 3A).

Treatment with agonistic anti–4-1BB mAb is known to enhance the proliferation of NK cells in vitro (18). However, the frequency of NK cells was very low in the secondary lymphoid organs, indicating that in vivo 4-1BB triggering induced apoptosis of peripheral NK cells (13), as well as reduced the influx of NK cells into the spleen by suppressing the generation of NK cells from BM. To test this hypothesis, we determined the proportions and numbers of NK cells in the BM of rat IgG- or anti–4-1BB mAb-treated B6 mice injected with EL4 cells, as described above. Total numbers of BM leukocytes increased 1.5–2-fold as the EL4 tumors grew (Fig. 3B, left panel). However, the proportion of NK

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**FIGURE 2.** Depletion of NK or CD8+ T cells. A and B, EL4 tumor-challenged B6 mice were treated with anti–4-1BB mAb or rat IgG, as previously described. Some mice were additionally injected i.p. with dCD8 (A) or dNK (B) on PI days 0, 5, 10, and 15. C and D, EL4 tumor-challenged WT (+/+) and 4-1BB−/− (−/−) B6 mice were injected with dCD8 on PI days 0, 5, 10 (C), and 15 or with dNK on PI days 10 and 15 (D). Volumes of tumor tissue in each mouse on PI day 21 (n = 5). Results are representative of three independent experiments. "p < 0.05; "p < 0.01; ""p < 0.001.

dCD8, depleting anti-CD8 mAb; dNK, anti-NK1.1 mAb.

**FIGURE 3.** Frequency of NK cells in spleen and BM. A, EL4 tumor-challenged B6 mice were injected i.p. with anti–4-1BB mAb or rat IgG, as previously described. Lymphocytes were prepared from spleens on PI day 21, stained with anti–CD8β-PE-Cy5 and anti–NK1.1-PE, and the proportions of NK1.1+ cells were determined (n = 5). Representative results of flow cytometric analyses (left panel) and graph of splenic NK cell percentages (right panel). B, BM cells were collected from tibias on PI day 21, counted, and stained with anti–NK1.1-PE and anti–CD3-PE-Cy5. Total numbers of BM cells (left panel), proportions of NK1.1+CD3− cells in BM (middle panel), and absolute numbers of NK1.1+CD3− cells in BM (right panel) were determined as described in Materials and Methods. Naive indicates WT B6 mice not challenged with EL4 cells. C and D, Splenocytes and BM (tibia) cells were collected from naive or EL4 tumor-challenged WT (+/+) and 4-1BB−/− (−/−) B6 mice on PI day 21, counted, and stained with anti–NK1.1-PE and anti–CD3-PE-Cy5. C, Proportions of NK1.1+CD3− cells in the spleen (n = 9). D, Absolute numbers of total BM cells (left panel), proportion of NK1.1+CD3− cells (middle panel), and absolute numbers of NK1.1+CD3− cells in BM (right panel) were determined (n = 4 for WT or n = 5 for 4-1BB−/− mice). "p < 0.05; ""p < 0.01; """"p < 0.001.
cells was slightly reduced by tumor growth, and it was significantly decreased by treating with anti–4-1BB mAb (Fig. 3B, middle panel). The absolute numbers of NK cells were also reduced (Fig. 3B, right panel).

The NK cell number was significantly reduced in 4-1BB−/− mice compared with WT mice, as noted previously (2). Although tumor challenge did not change the percentage of NK cells in WT mice, the NK cell frequency in tumor-bearing 4-1BB−/− mice was significantly (p = 0.0075) increased as the tumor grew (Fig. 3C). We noted that the number of NK cells was very variable among the tumor-challenged 4-1BB−/− mice (Fig. 3C). We believe that this is a reflection of the variation in tumor sizes observed in Fig. 1B, because 4-1BB−/− mice are spontaneously resistant to tumor growth. Total numbers of BM leukocytes increased 2-fold in both groups of mice (Fig. 3D, left panel); however, the proportion of NK cells decreased slightly in the WT mice after tumor challenge, whereas it increased in the 4-1BB−/− mice (Fig. 3D, middle panel). Eventually, the absolute number of BM NK cells increased nearly 3-fold in the tumor-challenged 4-1BB−/− mice, whereas it did not increase significantly in the WT mice (Fig. 3D, right panel). These results imply that although other immune cells in the BM of WT mice increase in response to tumor challenge, NK cells do not increase selectively. These findings are consistent with a previous report that tumor growth actively inhibits NK cell development in the BM, leading to escape from immune surveillance (19, 20).

When we plotted tumor growth versus NK1.1+CD3− cells in the BM, there was a clear inverse correlation between tumor volume and numbers of BM NK cells in WT and 4-1BB−/− mice but not in WT mice treated with anti–4-1BB mAb (Supplemental Fig. 1). Collectively, these results indicate that 4-1BB triggering reduces BM NK cell numbers and that NK cells are more important for preventing tumor growth in 4-1BB−/− mice.

**Blockade of NK cell development by 4-1BB signaling in BM**

Because the intensity of 4-1BB signaling and NK cell frequency were inversely correlated, we next analyzed the developmental stages of BM NK cells in the absence and presence of 4-1BB signaling, as well as after hyperactivation of 4-1BB signaling, to determine whether 4-1BB signaling is involved in NK cell development. NK cell development in the mouse BM can be divided into five stages: stage I: CD122+NK1.1−DX5− NK cell precursors; stage II: acquisition of NK1.1, αv, and CD94; stage III: acquisition of Ly49; stage IV: proliferation; and stage V: expression of CD11b and CD43 (21). There were no significant differences in stage I, II, and III cells between WT and 4-1BB−/− mice before and after tumor challenge (Supplemental Fig. 2). Naive WT and 4-1BB−/− mice retained similar levels of CD11b+ mature NK cell frequencies in the BM (Fig. 4A, left). However, the proportion of CD11b+ mature NK cells was reduced in WT mice 21 d after EL4 tumor challenge, consistent with previous reports (19, 20), but not in 4-1BB−/− mice (Fig. 4A, middle). These outcomes were not significantly changed by treatment with anti–4-1BB mAb (Fig. 4A, right). According to the forward light scatter (FSC) profiles, the majority of the gated NK1.1+CD3− NK cells were uniformly small in size, indicating that an influx of peripheral NK blasts was very minimal. Statistical analysis confirmed that the proportion of CD11b+ immature NK cells decreased significantly in the 4-1BB−/− mice, but not in the 4-1BB−/− mice, following tumor challenge (Fig. 4B).

The absolute numbers of CD11b− immature NK cells were comparable in the WT and 4-1BB−/− mice before tumor challenge, and they had increased 1.7-fold in the WT mice and 2.4-fold in the 4-1BB−/− mice 21 d after tumor injection (Fig. 4C). Despite the increase in total BM and CD11b− immature NK cell numbers, the number of CD11b+ mature NK cells did not increase at all in the WT mice, whereas they increased 1.4-fold in the 4-1BB−/− mice (Fig. 4C). Treatment with anti–4-1BB mAb resulted in severe reductions in CD11b− immature and CD11b+ mature NK cell numbers in the BM (Fig. 4D).

Therefore, we conclude that moderate 4-1BB signaling mainly reduces CD11b+ mature NK cell numbers, but not CD11b− NK cells numbers, by moderately blocking the conversion of CD11b− immature NK cells into CD11b+ mature NK cells, and strong 4-1BB triggering reduces CD11b− and CD11b+ NK cell numbers in

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** 4-1BB–mediated inhibition of NK cell development in the BM. EL4 tumor-challenged WT (+/+ ) and 4-1BB−/− (−/−) B6 mice were treated with rat IgG or anti–4-1BB mAb, as described above. On PI day 21, BM cells were counted and stained with anti–CD3-PE-Cy5, anti-NK1.1-PE, and anti–CD11b-FTC. A, NK1.1+CD3− NK cells were gated and plotted as FSC versus CD11b. B, Proportions of CD11b− and CD11b+ NK cells in BM. C, Absolute numbers of CD11b− and CD11b+ NK cells in BM before and after EL4 tumor challenge. D, Absolute numbers of CD11b− and CD11b+ NK cells in BM after treatment with rat IgG or anti–4-1BB mAb. *p < 0.05; **p < 0.01.
BM. Therefore, the absence of 4-1BB signaling markedly increases the number and frequency of NK cells in BM of tumor-bearing mice.

**IFN-γ mediates the inhibition of NK development by 4-1BB**

Previous work indicated that overexpression of IFN-γ impairs NK cell development (22) and that NK cell development is affected by soluble factors associated with tumor growth (19, 20). Because 4-1BB triggering severely affects immune responses as a result of the massive induction of IFN-γ (13, 23, 24), 4-1BB signaling might modulate NK cell development through IFN-γ. To assess the involvement of IFN-γ in the 4-1BB-mediated inhibition of NK cell development, GKO and GRKO mice were challenged s.c. with EL4 cells and monitored for tumor growth and NK development. As expected, tumor cells grew more rapidly in the GKO and GRKO mice than in WT mice (Supplemental Fig. 3A). Nevertheless, when we measured BM NK cell frequencies 18 d after tumor injection, their frequencies were not severely decreased (Supplemental Fig. 3B). Naïve C57BL/6, 4-1BB−/−, GKO, and GRKO mice had comparable levels (∼55%) of CD11b+ mature NK cells in their BM before tumor challenge (data not shown); these decreased to 23.61 ± 1.74% in WT mice, 43.24 ± 2.22% in 4-1BB−/− mice, 39.47 ± 2.89% in GKO mice, and 39.87 ± 1.85% in GRKO mice after chronic tumor growth (Fig. 5A). In the absolute numbers, CD11b+ NK cells of GKO or GRKO mice were not suppressed by tumor growth (Fig. 5B). Consequently, the frequencies of peripheral NK cells in tumor-draining lymph nodes increased in the absence of IFN-γ/IFN-γR signaling 18 d after tumor challenge (Fig. 5C).

To examine the link between 4-1BB signaling and IFN-γ, WT and 4-1BB−/− mice were challenged s.c. with EL4 cells and then infected i.v. with Ad–IFN-γ (or Ad-lacZ as a control) 10 d after tumor injection. Infection with Ad–IFN-γ, but not with Ad-lacZ, led to decreased CD11b+ mature NK cell frequencies in WT and 4-1BB−/− mice on PI day 21 (Fig. 5D). Statistical analysis confirmed that the proportion of CD11b+ mature NK cells declined significantly in the 4-1BB−/− mice (from 40.5 ± 1.36% to 24.5 ± 1.5%) but not in the WT mice (from 28.1 ± 3.6% to 23.9 ± 1.5%) (Fig. 5E).

Next, we treated EL4-challenged WT and GRKO mice with anti-4-1BB mAb or rat IgG and analyzed the frequencies of NK cells in spleen and BM. Interestingly, the proportion of splenic NK cells was decreased by ∼90% in WT mice and by ∼70% in GRKO mice by 4-1BB triggering, which implies that IFN-γ may be partially responsible for the reduction in peripheral NK cells (Fig. 5F, left panel, Supplemental Fig. 4A). 4-1BB triggering reduced NK cells in WT and GRKO mice, but the reduction was more severe in the WT mice (Supplemental Fig. 4B). However, BM NK cells were reduced by 4-1BB triggering in WT mice but not in GRKO mice (Fig. 5F, right panel, Supplemental Fig. 4A). These results demonstrate that 4-1BB triggering mediates the inhibition of NK cell development in the BM via IFN-γ.

**4-1BB–mediated inhibition of NK cell development in the absence of T and B cells**

T and NK cells are the major cell types that produce IFN-γ (14, 25), and it is well-known that massive expansion of CD8+ T cells and high levels of IFN-γ production are the most prominent features of

![FIGURE 5](http://www.jimmunol.org/) Normal development of NK cells in IFN-γ− and IFN-γR−/−deficient mice. A–C, WT, 4-1BB−/−, GKO, and GRKO B6 mice were injected s.c. with EL4 tumor cells. Lymphocytes were collected from tibias and TDLNs on PI day 18 and stained with fluorescence-labeled anti-CD3, anti-NK1.1, and anti-CD11b mAb. A, Proportions of NK1.1+CD3+CD11b+ and NK1.1+CD3+CD11b− NK cells in BM NK cells. B, Absolute numbers of NK1.1+CD3+CD11b+ and NK1.1+CD3+CD11b− NK cells in BM of WT, 4-1BB−/−, GKO, and GRKO B6 mice. C, Percentages of NK1.1+CD3− cells in TDLNs. D–F, WT and 4-1BB−/− B6 mice were injected i.v. with Ad-lacZ or Ad–IFN-γ 10 d after EL4 tumor injection. On PI day 21, BM cells were stained with fluorescence-labeled anti-CD3, anti-NK1.1, and anti-CD11b mAb. D, NK1.1+CD3− NK cells were gated and plotted as FSC versus CD11b. E, Proportions of NK1.1+CD3+CD11b+ and NK1.1+CD3+CD11b− NK cells in BM NK cells. F, EL4 tumor-challenged WT and GRKO mice were treated i.p. with anti-4-1BB mAb or rat IgG on PI days 5, 10, 15, and 20. NK1.1+CD3+ cells in spleen and BM were analyzed by flow cytometry on PI day 21 (n = 5). Plotted data are mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.
in vivo 4-1BB triggering (23). Therefore, we can readily suppose that CD8+ T cells are primarily responsible for IFN-γ production by 4-1BB triggering (13). However, it was not clear whether NK cells are also involved in the IFN-γ production. We generated 4-1BB+/+ RAG2−/− (DKO) mice that lacked T, B, and NKT cells, as well as 4-1BB signaling, and examined tumor growth and NK cell development after MC38 tumor injection. Tumor growth was suppressed in the 4-1BB−/− mice, as expected, but not in the WT, RAG2−/−, or DKO mice, which indicates that CD8+ T cells are required to suppress tumor growth in the 4-1BB−/− mice (Fig. 6A). BM NK cell development was normal in RAG2−/− and DKO mice before tumor challenge, but it was blocked by tumor growth only in the RAG2−/− mice (Fig. 6B, Supplemental Fig. 5A) (19).

When we analyzed several cytokines in sera collected from DKO and RAG2−/− mice 7 d after tumor challenge, IFN-γ was significantly lower in the DKO mice (Fig. 6C). Because T and NK cells are the major producers of IFN-γ (14, 25), the residual IFN-γ seemed to be produced by NK cells. Although IFN-γ was responsible for the suppression of NK cell development, it was possible that IFN-γ-R expression differed on WT and 4-1BB−/− NK cells in the BM or that NK cell development was directly affected by anti-4-1BB mAb due to 4-1BB expression on BM NK cells. However, we found no difference in the expression of IFN-γ-R on BM NK cells from WT and 4-1BB−/− mice after tumor injection and confirmed that BM NK cells did not express substantial amounts of 4-1BB on their surface (Fig. 6D).

To determine whether the 4-1BB signaling in periphery was sufficient to suppress NK cell development in BM, CD8+ T cells from WT and 4-1BB−/− mice were adoptively transferred into 4-1BB−/− RAG2−/− B6 mice, to generate the condition that only peripheral CD8+ T cells express 4-1BB, and they were subsequently challenged with MC38 tumor cells. Two weeks after the tumor challenge, total numbers of BM cells were 0.9 ± 0.24 × 106 cells in 4-1BB+/− CD8+ T cell-transferred mice and 1.3 ± 0.39 × 106 cells in 4-1BB−/− CD8+ T cell-transferred mice (data not shown). The proportion of NK cells in the BM decreased to 10% of WT levels following transfer of 4-1BB+/− CD8+ T cells but not 4-1BB−/− CD8+ T cells, although there were comparable levels of CD8+ T cells (Fig. 6E); this indicates that NK cell development in the BM was blocked by 4-1BB–triggered peripheral CD8+ T cells.

Taken together, these results suggest that 4-1BB signaling in the periphery inhibits NK cell development in the BM by increasing IFN-γ production from T cells and NK cells. 4-1BB deficiency severely dampened the in vivo expression of IFN-γ that was needed to induce and maintain MHC class I expression on tumor cells. Therefore, tumor cells may have more opportunities to downregulate MHC class I in 4-1BB−/− mice, which would mean that the cytolytic activities of NK cells would be more important in the antitumor response of 4-1BB−/− mice. Nevertheless, tumor growth was not suppressed at all in 4-1BB−/− RAG2−/− DKO mice (Fig. 6A) or CD8+ T cell-depleted 4-1BB−/− mice (Fig. 2C). Because the NK cells from WT and 4-1BB−/− mice had comparable cytotoxicity (2), we concluded that the enhanced antitumor response of 4-1BB−/− mice was primarily due to the noncytolytic effects of NK cells. Our findings also suggest that the antitumor immunity of the host switches the dominant immune cell-type from innate NK cells to adaptive CD8+ T cells as a result of 4-1BB–mediated IFN-γ production.

**Discussion**

We have shown that 4-1BB signaling in the periphery negatively regulates NK cell development in the BM by increasing IFN-γ production. Thus, in mouse tumor models, NK cell development in the BM was enhanced in the absence of normal 4-1BB signaling, moderately decreased in the presence of normal 4-1BB signaling, and severely suppressed by 4-1BB agonists. NK cells proved to be required for the antitumor activities of CD8+ T cells in the 4-1BB−/− mice. Therefore, the antitumor effects shown in 4-1BB−/− mice seem to be the result of the enhanced NK numbers and activities.

IFN-γ, the crucial factor in 4-1BB–mediated suppression of NK cell development, is known to disrupt the development of B cells, NK cells, and NKT cells (22), to cause activation-induced cell death of effector T cells (26), and to negatively regulate self-renewal of hematopoietic stem cells (27). In IFN-γ transgenic mice (165 ± 47 pg/ml of IFN-γ in serum), there were severe B cell lineage reductions, T cell lineage alterations, and hematopoietic progenitor deficiencies, as well as reduced NK cells in spleen and BM (22, 28). Young et al. (28) reported that elevated IFN-γ reduced numbers of NK progenitors in the BM and inhibited maturation of NK cells and hematopoietic stem cell proliferation. These findings are consistent with ours because 4-1BB triggering increased IFN-γ in vivo; typically suppressed CD4+ T, B, and NK

![FIGURE 6](https://www.jimmunol.org/)

**FIGURE 6.** NK cell development in tumor-bearing RAG2−/− and 4-1BB−/− RAG2−/− mice. WT, 4-1BB−/−, RAG2−/−, and 4-1BB−/− RAG2−/− (DKO) B6 mice were injected s.c. with MC38 tumor cells. A, Volumes of tumor tissue on PI day 21 (n = 5). B, BM cells were stained with fluorescence-labeled anti-CD3, anti-CD11b, and anti-NK1.1 mAb. NK1.1+CD3− cells were gated, and the proportion of CD11b+ and CD11b− cells was determined. C, Sera were collected from RAG2−/− and DKO B6 mice 7 d after tumor challenge, and serum cytokine levels were determined by flow cytometry using a Cytometric Bead Array kit (BD Biosciences). D, BM cells from naive RAG2−/− and DKO B6 mice were stained with anti–4-1BB-PE or anti–IFN-γ-PE, as well as anti–NK1.1-PE-Cy5. Plotted data are mean ± SD. E, CD8+ T cells isolated from LNs and spleens of WT and 4-1BB−/− B6 mice, adoptively transferred into 4-1BB−/− RAG2−/− mice at 1 × 106 cells per mouse, and challenged s.c. with MC38 tumor cells. Two weeks after the injection of MC38 tumor cells, lymphocytes from BM of each group of mice were isolated and stained with fluorescence-labeled anti-CD8 and anti-NK1.1 mAb. *p < 0.05; **p < 0.01.
cells; increased CD8+ T cells; and reduced numbers of BM and thymus cells (13, 29).

4-1BB triggering generally increases IFN-γ production in vivo, and this can reach ~1 ng/ml of serum (24). Moreover, the serum level of IFN-γ was 84.34 ± 15.2 pg/ml in tumor-challenged RAG2−/− mice, which lack T and NKT cells, and it decreased to 10–20 pg/ml in the absence of 4-1BB signaling (Fig. 6C). These data indicate that enough IFN-γ is produced by tumor-sensitized NK cells in the presence of endogenous 4-1BB signaling to modulate NK cell development in the BM. Collectively, anti–4-1BB mAb-treated mice display similar immune cell profiles to IFN-γ transgenic mice, such as reduction of B and NK cells, alteration of the T cell ratio, and fewer immune cells in secondary lymphoid organs and BM. These observations support the hypothesis that 4-1BB signaling in the periphery negatively regulates NK cell development and is mediated by increasing IFN-γ.

Lee et al. (13) suggested that 4-1BB triggering suppresses B cell development IFN-γ dependently, whereas the development of NK and NKT cells is inhibited via T cells in an IFN-γ-independent manner. They demonstrated suppression of NK cells in irradiated RAG1−/− mice 2 wk after they were reconstituted with BM cells from WT or IFN-γ−/− mice in the presence of anti-4-1BB stimulation but in the absence of antigenic stimulation. In this study, we examined the effects of 4-1BB signaling on NK cell development in tumor models and concluded that the 4-1BB-mediated suppression of NK cells was IFN-γ dependent. There seem to be two explanations for the different views of the role of IFN-γ in 4-1BB-mediated suppression of NK cell development. First, they could be due to the different immune responses studied: antitumor responses (our work) versus normal hematopoiesis (13). Alternatively, the chimeras would contain WT or IFN-γ−/− T cells as a result of the transfer of WT or IFN-γ−/− BM cells, they might regenerate normal NK cells from the host (RAG1−/−) BM. In particular, because NK cells only need ~1 wk to develop from BM, and T cells require ~3 wk (30, 31), NK cells might have been fully regenerated in the chimeras because the mice were only treated with anti–4-1BB mAb 2 wk after the irradiation. In addition, because endogenous 4-1BB signaling in RAG2−/− mice is sufficient to inhibit NK cell development in the BM (Fig. 6), the chimeras receiving anti–4-1BB mAb should produce sufficient IFN-γ from the NK cells to block NK cell development in the BM. We found that only moderate numbers of NK cells were recovered in the spleen of tumor-challenged IFN-γ−/− knockout mice after 4-1BB-mediated suppression, whereas they recovered fully in the BM (Fig. 5D). In addition, in the steady state, 4-1BB−/− mice had reduced numbers of NK cells in secondary lymphoid organs but normal numbers in the BM, whereas in tumor-challenged 4-1BB−/− mice, NK cells increased in secondary lymphoid organs and BM (Fig. 3C, 3D). In vitro culture conditions, 4-1BB trig-
erging enhanced the proliferation of NK cells (Supplemental Fig. 5B), as previously reported (18). Therefore, different mechanisms may regulate peripheral NK cell and BM NK cell development.

Our findings suggest that 4-1BB signaling in the periphery including CD8+ T and NK cells negatively regulates NK cell development in BM; thus, antitumor immunity is enhanced in 4-1BB−/− mice as a result of the increase in NK cells, whose function seems to be to promote CD8+ T cell activities. A recent finding demonstrates a species difference between the response of human and murine NK cells to 4-1BB signaling (32). Nevertheless, because the 4-1BB-mediated suppression of NK cell development in BM primarily depends on the IFN-γ from activated CD8+ T cells, the effects of 4-1BB triggering on NK cell development in BM will be similar between humans and mice. Because it is likely that agonistic Ab to 4-1BB can have adverse side effects during long-term treatment in the clinic as the result of excessive IFN-γ production, anti–4-1BB mAb therapy should be combined with a strategy to maintain IFN-γ at an optimal level.

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