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4-1BB Promotes the Survival of CD8⁺ T Lymphocytes by Increasing Expression of Bcl-x_L and Bfl-1¹

Hyeon-Woo Lee,* Su-Jung Park,* Beom K. Choi,* Hyun Hwa Kim,* Kyung-Ok Nam,* and Byoung S. Kwon^{2,*†}

4-1BB, a T cell costimulatory receptor, prolongs CD8⁺ T cell survival. In these studies, 4-1BB stimulation was shown to increase expression of the antiapoptotic genes *bcl-x_L* and *bfl-1* via 4-1BB-mediated NF-κB activation. This signaling pathway was specifically inhibited by PDTC and was different from the pathways that enhanced CD8⁺ T cell proliferation. The results suggest a role for the antiapoptotic activities of Bcl-x_L and Bfl-1 proteins in 4-1BB-mediated CD8⁺ T cell survival in vivo. *The Journal of Immunology*, 2002, 169: 4882–4888.

The T cell costimulatory receptor 4-1BB provides a survival signal to T cells against activation-induced cell death (1) and to superantigen-activated CD8⁺ T cells to prevent clonal deletion after exposure to superantigen. This occurs independently of adjuvant or cytokines (2), indicating that 4-1BB-mediated signaling directly prolongs CD8⁺ T cell survival. CD8⁺ T cell survival mediated by 4-1BB appears to be different from the effects of CD28 costimulation, which are characterized by clonal expansion and possibly enhanced short-term survival (2–4).

Several molecules that play key roles in regulating apoptosis in T cells have been identified (5), including members of the Bcl-2 family. Bcl-2-related proteins have either antiapoptotic effects (e.g., Bclw, Bcl-2, Bcl-x_L, and Bfl-1) or proapoptotic effects (e.g., Bax, Bak, and Bad). Bcl-x_L has been shown to protect cells from apoptosis evoked by a variety of agents that also activate NF-κB (6, 7). Khoshnan et al. (8) demonstrated that CD3/CD28-mediated activation of NF-κB and up-regulation of Bcl-x_L expression inhibited apoptosis in human CD4⁺ T cells. In B lymphocytes, CD40-mediated cell survival required an NF-κB-dependent increase in Bcl-x_L and Bfl-1 expression (9). Also, Akt mediated survival in CD4⁺ and CD8⁺ double-positive thymocytes and mature T cells through the regulation of NF-κB and Bcl-x_L (10).

Although studies to date have shown that 4-1BB produces signals through TNFR-associated factor–NF-κB-inducing kinase–NF-κB (11, 12) and TNFR-associated factor–apoptosis signal-regulating kinase–p38 mitogen-activated protein kinase (MAPK)³ or stress-activated protein kinase/c-Jun N-terminal kinase pathways

(13, 14), 4-1BB-mediated signal transduction pathways specific for T cell survival have not yet been defined. To elucidate the molecular mechanisms by which 4-1BB promotes CD8⁺ T cell survival, we have examined expression of antiapoptotic genes and the signal transduction pathways from 4-1BB to Bcl-x_L in CD8⁺ T cells. In this study, we show that 4-1BB induces expression of the survival genes *bcl-x_L* and *bfl-1* through activation of NF-κB.

Materials and Methods

Mice, reagents, and Abs

Male BALB/c mice were obtained from Harlan (Indianapolis, IN). Animals were maintained under specific pathogen-free conditions. Anti-CD3 mAb (145-2C11 clone), biotin-labeled and PE-labeled anti-CD8 mAb, isotype control Ab, and an apoptosis detection kit were purchased from BD Pharmingen (San Diego, CA). Agonistic anti-4-1BB mAbs (3H3 and 3E1) were kindly provided by Dr. R. S. Mittler (Emory University, Atlanta, GA). Streptavidin-conjugated microbeads and LS columns were purchased from Miltenyi Biotec (Auburn, CA). Cycloheximide (CHX) was purchased from Sigma-Aldrich (St. Louis, MO). LY294002, 1-pyrrolidinedithioic acid, ammonium salt (PDTC), SB203580, U0126, and PD98059 were purchased from Calbiochem-Novabiochem (La Jolla, CA). All Abs for Western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NF-κB consensus and mutant probes for EMSA were also purchased from Santa Cruz Biotechnology.

CD8⁺ T cell purification

Cell suspensions were prepared from the spleens and lymph nodes of BALB/c mice. Cells were incubated at 37°C for 1 h in flasks to eliminate adherent cells before purification. CD8⁺ T cells were purified using the MACS magnetic separation system according to the manufacturer's instructions (Miltenyi Biotec). In brief, cells were resuspended at a concentration of 10⁸ cells/ml in PBS containing 5% FBS, incubated with anti-CD8 mAb conjugated with biotin, and collected by incubating with streptavidin microbeads at 4°C for 15 min. LS columns (Miltenyi Biotec) were used for the selection of CD8⁺ T cells. CD8⁺ T cell purity was routinely shown to be >95% by flow cytometry.

T cell stimulation

Purified CD8⁺ T cells were plated at 10⁶ cells/well in 96-well round-bottom plates with 0.5 μg/ml anti-CD3 mAb (BD Pharmingen) for 16 h. After incubation, cells were stained with anti-4-1BB-FITC (3E1-FITC); >70% of the cells routinely showed cell surface expression of 4-1BB by flow cytometry. After 4-1BB expression on the purified CD8⁺ T cells was verified, the cells were preincubated with or without various pharmacological inhibitors for 1 h, and then with 5 μg/ml agonistic anti-4-1BB mAb (3H3) or rat IgG2a as an isotype control for the indicated periods.

*Immunomodulation Research Center, University of Ulsan, Ulsan, Korea; and †Louisiana State University Eye Center, Louisiana State University Health Sciences Center School of Medicine, New Orleans, LA 70112

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² Address correspondence and reprint requests to Dr. Byoung S. Kwon, Immunomodulation Research Center, University of Ulsan, Ulsan, Korea 680-749. E-mail address: bskwon@mail.ulsan.ac.kr

³ Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; CHX, cycloheximide; PDTC, 1-pyrrolidinedithioic acid; PI3K, phosphatidylinositol-3 kinase; MEK, MAPK kinase; ERK, extracellular signal-related kinase.

T cell proliferation assay

Purified CD8⁺ T cells were plated at 5×10^5 cells/well in 96-well round-bottom plates and stimulated as described above. During the final 12 h of culture, the cells were pulsed with 1 μ Ci/well [³H]thymidine (NEN, Boston, MA). Cellular DNA was harvested and counted by liquid scintillation spectroscopy.

Apoptosis assay

Anti-CD3-treated CD8⁺ T cells were stimulated with 5 μ g/ml 3H3 or rat IgG2a for various times. Apoptosis was evaluated by flow cytometric detection of phosphatidylserine expression after the addition of FITC-labeled Annexin V (BD Pharmingen) and propidium iodide.

Western blotting

Purified CD8⁺ T cells were stimulated as described above and proteins were extracted with lysis buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na₃VO₄, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor mixture). Equal amounts of protein from each sample were diluted with 4 \times SDS sample buffer, applied to SDS-PAGE gels, separated, and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Each protein of interest was detected with primary Abs and secondary Ab-HRP. Bound Abs were detected by ECL (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

RNase protection assay

Five- to 10- μ g samples of total RNA extracted (RNAwiz; Ambion, Austin, TX) from anti-CD3 mAb-treated or anti-CD3 mAb plus anti-4-1BB mAb-treated CD8⁺ T cells were subjected to an RNase protection assay as specified by the manufacturer, using the mAPO-2 probe set (BD Pharmingen). The resulting protected RNAs were resolved on 5% denaturing polyacrylamide gels and exposed to x-ray film.

EMSA

EMSA analysis was conducted as previously described (15). Briefly, CD8⁺ T cells were harvested and washed with PBS containing 1 mM Na₃VO₄

and 5 mM NaF. The cells were then incubated for 5 min on ice in 1 ml of lysis buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 0.2% Nonidet P-40. After centrifugation at 3000 rpm for 5 min, the pellets were further treated for 30 min at 4°C with 40 μ l of lysis buffer supplemented with 420 mM NaCl and 20% glycerol and then subjected to centrifugation at 13,000 rpm for 10 min. The resulting supernatant was used as the nuclear extract in the EMSA analysis. Next, 4 μ g of nuclear extract and \sim 0.5 ng of labeled oligonucleotides were incubated for 20 min at room temperature with 1 μ g of poly(dI-dC) in 20 μ l of a binding buffer containing 13 mM HEPES (pH 7.9), 65 mM NaCl, 0.15 mM EDTA, 8% glycerol, and 1 mM DTT. The complexes formed were separated from the free probe by electrophoresis in a 5% nondenaturing polyacrylamide gel containing 0.5% Tris-borate EDTA, and the gel was dried and exposed to x-ray film. Double-stranded synthetic oligonucleotides corresponding to the consensus NF- κ B binding element (top strand, AGTTGAGGGGACTTCCAGG) and its mutant element (top strand, AGTTGAGGCGACTTCCAGG) were labeled with [γ -³²P]ATP by means of polynucleotide kinase and used as probes for the κ B site.

Results

4-1BB ligation enhances proliferation and survival of CD8⁺ T lymphocytes

Approximately 70% of purified CD8⁺ T lymphocytes expressed 4-1BB 16 h after treatment with 0.5 μ g/ml anti-CD3 mAb (Fig. 1A). Stimulation of CD8⁺ T lymphocytes with both anti-CD3 and anti-4-1BB mAbs enhanced proliferation by approximately threefold, compared with treatment with anti-CD3 mAb alone (Fig. 1B). 4-1BB-mediated CD8⁺ T cell proliferation was blocked by an NF- κ B inhibitor (PDTC; 20 μ g/ml), a phosphatidylinositol-3 kinase (PI3K) inhibitor (LY294002; 20 μ M), and two MAPK kinase (MEK) inhibitors (5 μ M U0126 and 30 μ M PD98059) (Fig. 1C). By contrast, a p38 MAPK inhibitor (SB203580; 20 μ M) had no

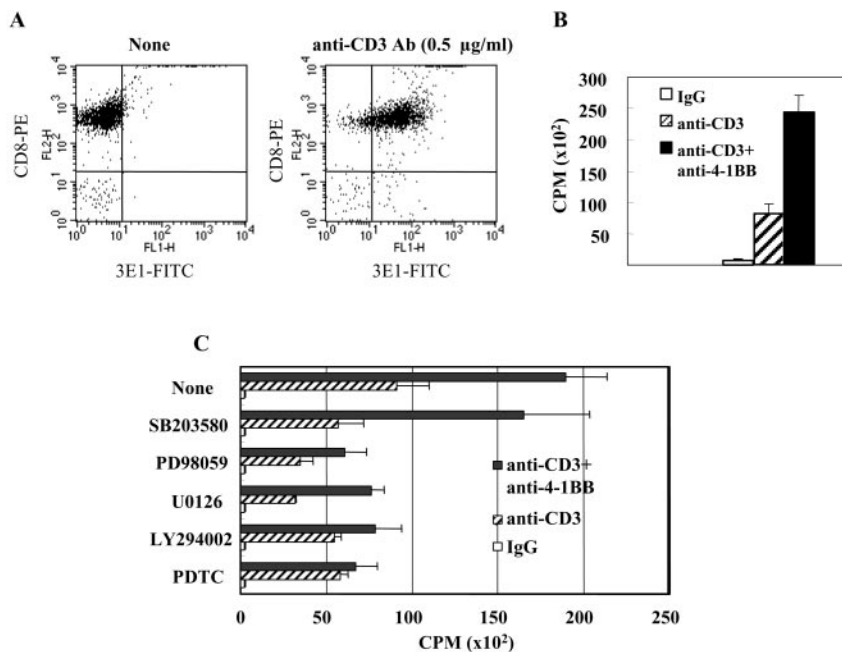


FIGURE 1. CD8⁺ T lymphocytes (2×10^5 – 5×10^5) purified from lymph nodes and spleens of BALB/c mice using the MACS magnetic separation system were plated in a round-bottom 96-well microplate. The cells were incubated with 0.5 μ g/ml anti-CD3 mAb or isotype control IgG for 16 h. **A**, 4-1BB expression on purified murine primary CD8⁺ T cells induced by anti-CD3 Ab treatment. The cells were harvested, double stained with anti-CD8-PE and anti-4-1BB-FITC, and then analyzed by flow cytometry. **B**, Treatment with anti-4-1BB Ab enhances proliferation of CD8⁺ T cells. After 4-1BB expression was verified, the cells were treated with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 48 h. During the last 12 h of culture, the cells were pulsed with 1.0 μ Ci/well [³H]thymidine. All cells were harvested and counted by liquid scintillation spectroscopy. The results are represented as means \pm SD of triplicates. Similar results were obtained in at least six independent experiments. **C**, 4-1BB-induced enhancement of proliferation is blocked by PDTC, LY294002, U0126, and PD98059. After incubation with anti-CD3 mAb for 16 h, the cells were pretreated with vehicle, 20 μ M SB203580, 30 μ M PD98059, 5 μ M U0126, 20 μ M LY294002, or 20 μ g/ml PDTC for 1 h and then with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 48 h. During the last 12 h of culture, the cells were pulsed with 1.0 μ Ci/well [³H]thymidine. All cells were harvested and counted by liquid scintillation spectroscopy. The results are represented as means \pm SD of triplicates. Similar results were obtained in three independent experiments.

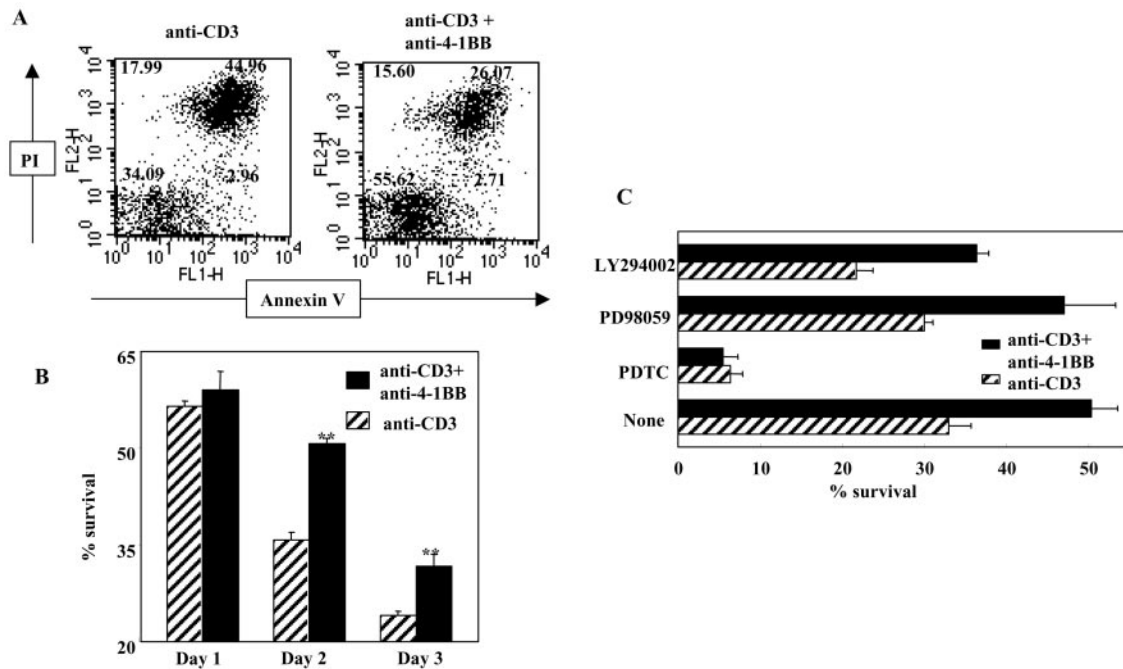


FIGURE 2. Treatment with anti-4-1BB mAb enhances survival of CD8⁺ T cells. **A**, The percentage of cells surviving at various time points was quantitated by flow cytometric detection using gating of the double-negative portion of dual-color Annexin V-FITC/propidium iodide staining. FACS profiles on day 2 are shown. **B**, The percentages of cells surviving on days 1, 2, and 3 of incubation are shown. The results are expressed as means \pm SD of triplicates. The asterisks indicate statistically significant differences compared with the anti-CD3 mAb-treated T cells (**, $p < 0.01$, Student's t test). Similar results were obtained in three independent experiments. **C**, Percent survival was measured at 48 h after treatment with anti-4-1BB in the presence or absence of inhibitors. The results are expressed as means \pm SD of triplicates. Similar results were obtained in two additional experiments.

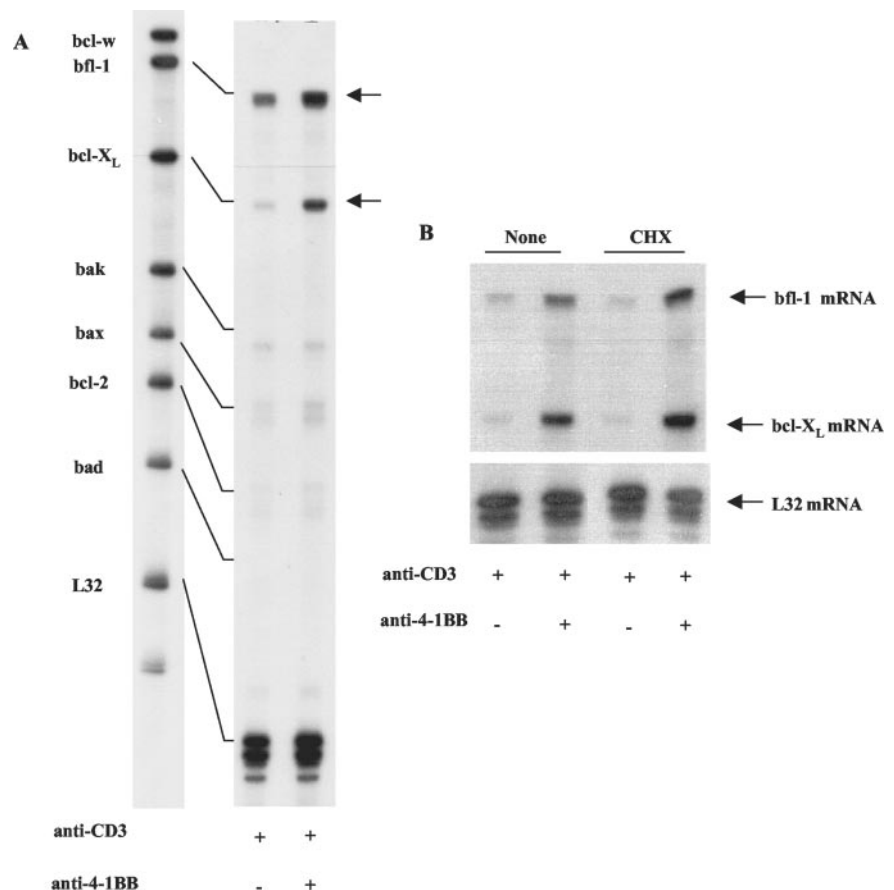
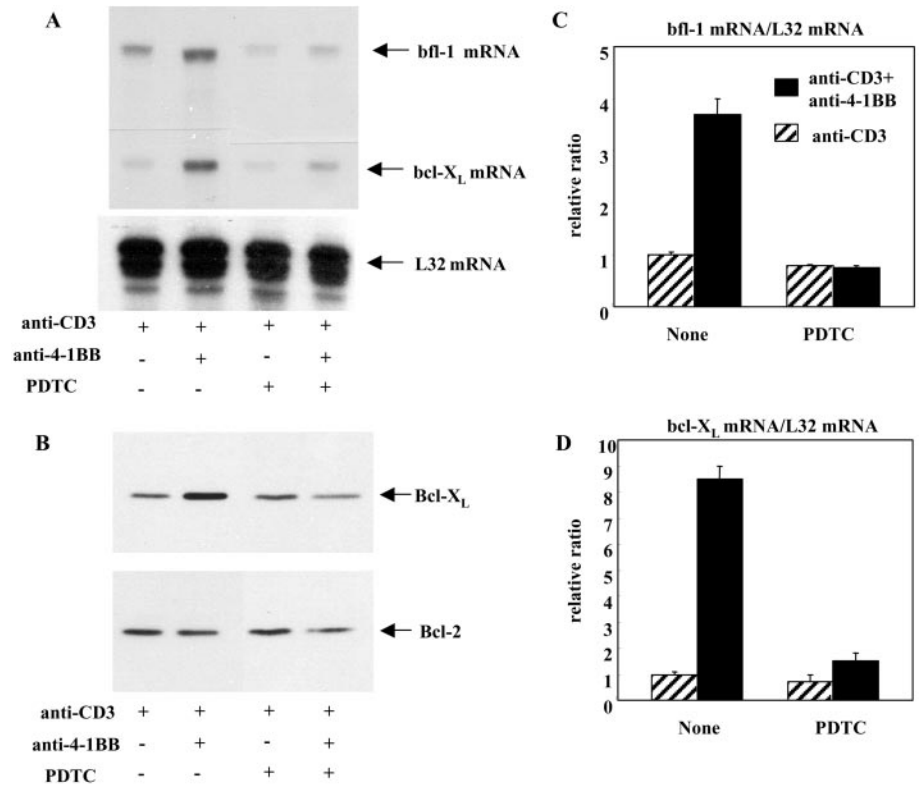


FIGURE 3. **A**, Treatment of CD8⁺ T cells with anti-4-1BB mAb up-regulates expression of *bcl-x_L* and *bfl-1* mRNA. Anti-CD3 mAb-treated CD8⁺ T cells were incubated with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 6 h. The cells were lysed to extract total RNA. Equal amounts (9 μ g) of total RNA were subjected to an RNase protection assay using the mAPO-2 probe set. Similar results were obtained in three independent experiments. **B**, 4-1BB-mediated up-regulation of *bcl-x_L* transcript is independent of de novo protein synthesis. Anti-CD3 mAb-treated CD8⁺ T cells were preincubated with or without 20 μ g/ml CHX for 1 h, then with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 6 h. An RNase protection assay was performed as described in **A**. Similar results were obtained in three independent experiments.

FIGURE 4. A, PDTC, an NF- κ B inhibitor, blocks 4-1BB-induced up-regulation of *bcl-x_L* and *bfl-1* mRNA expression. B, PDTC inhibits 4-1BB-induced up-regulation of Bcl-*x_L* protein, but it does not alter the level of Bcl-2 protein. Anti-CD3 mAb-treated CD8⁺ T cells were preincubated with vehicle or 20 μ g/ml PDTC (A and B) for 1 h, then with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 6 h. For RNase protection assays of *bcl-x_L* and *bfl-1* mRNA (A), total RNA was isolated after the cells were incubated with anti-4-1BB mAb or isotype control IgG for 6 h. For Western blotting analysis of Bcl-*x_L* and Bcl-2 protein (B), proteins were extracted from CD8⁺ T cells after a 24-h incubation with anti-4-1BB mAb or isotype control IgG. Similar results were obtained in three independent experiments. C, Densitometric measurements are presented as the relative abundance of *bfl-1* mRNA, compared with control L32 mRNA. D, Densitometric measurements are presented as the relative abundance of *bcl-x_L* mRNA, compared with control L32 mRNA. Values in C and D are means \pm SD from three experiments.



effect (Fig. 1C). In this study, inhibitors were added after a 16-h incubation with anti-CD3 mAb and confirmation of 4-1BB expression, followed by incubation with anti-4-1BB mAb. The data suggest that 4-1BB-mediated NF- κ B, MEK, and PI3K signaling path-

ways may be involved in 4-1BB-evoked proliferation. PD98059 and U0126 produced greater inhibition of anti-CD3-mediated CD8⁺ T cell proliferation, compared with the other inhibitors tested. The greater effect of these two compounds may be the result

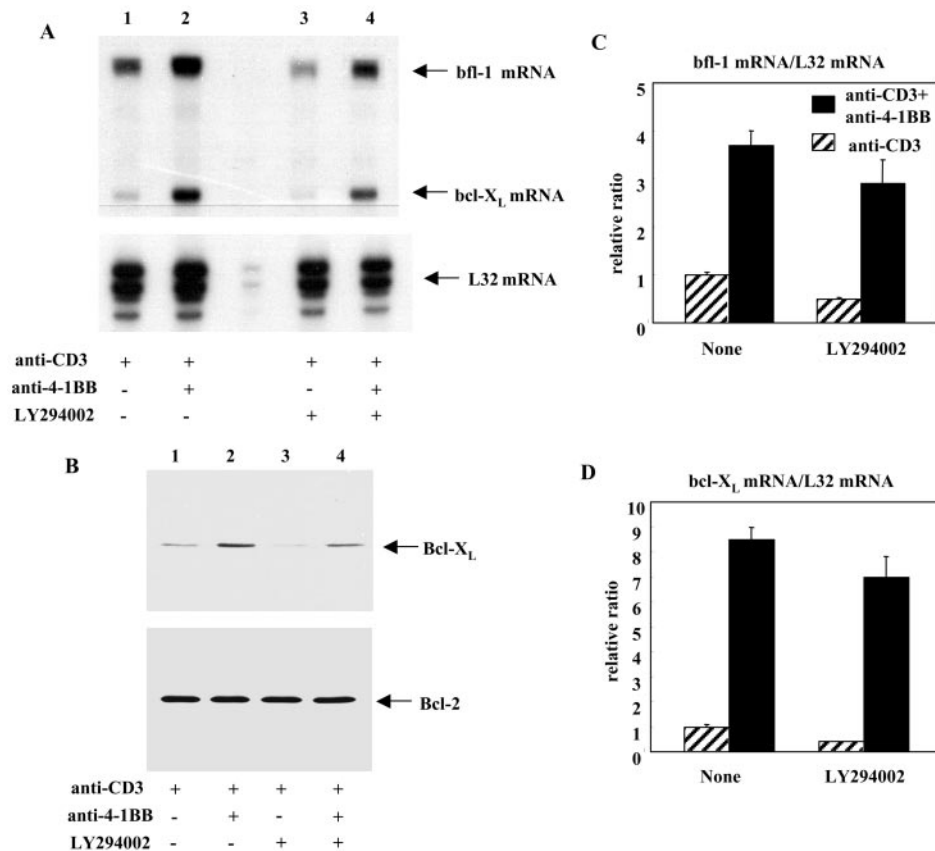


FIGURE 5. A, LY294002, a PI3K inhibitor, does not block 4-1BB-induced up-regulation of *bcl-x_L* and *bfl-1* mRNA expression. B, LY294002 does not block 4-1BB-induced up-regulation of Bcl-*x_L* protein. Anti-CD3 mAb-treated CD8⁺ T cells were preincubated with vehicle or 20 μ M LY294002 (A and B) for 1 h, then with 5 μ g/ml anti-4-1BB mAb or isotype control IgG. For RNase protection assays of *bcl-x_L* and *bfl-1* mRNA (A), total RNA was isolated after the cells were incubated with anti-4-1BB mAb or isotype control IgG for 6 h. For Western blotting analysis of Bcl-*x_L* and Bcl-2 protein (B), proteins were extracted from CD8⁺ T cells after a 24-h incubation with anti-4-1BB mAb or isotype control IgG. Similar results were obtained in three independent experiments. C, Densitometric measurements are presented as the relative abundance of *bfl-1* mRNA, compared with control L32 mRNA. D, Densitometric measurements are presented as the relative abundance of *bcl-x_L* mRNA, compared with control L32 mRNA. Values in C and D are means \pm SD from three experiments.

of inhibition of the TCR signaling pathway, rather than the 4-1BB pathway, or it may be that these two inhibitors affect both TCR and 4-1BB signaling pathways.

Percent survival of the cells was assessed at various time points by flow cytometric detection using gating of the double-negative portion of dual-color Annexin V-FITC/PI staining. Fig. 2A shows a FACS profile on day 2 of treatment with anti-CD3 or anti-CD3 plus anti-4-1BB. When CD8⁺ T cells were incubated with anti-4-1BB plus anti-CD3, percent survival was 15% greater on day 2 and 10% greater on day 3, compared with cells incubated with anti-CD3 alone (Fig. 2B). These results indicate that 4-1BB-mediated expansion of the CD8⁺ T cell population may result from both T cell proliferation and T cell survival. We tested the effects of inhibitors on 4-1BB-mediated cell survival (Fig. 2C). PDTC blocked the 4-1BB-mediated increase in cell survival, whereas LY294002 and PD98059 did not. It is noteworthy that LY294002 and PD98059 inhibited 4-1BB-induced cellular proliferation, as shown in Fig. 1C, whereas they had no effect on cell survival (Fig. 2C).

4-1BB ligation up-regulates expression of the anti-apoptotic genes *bcl-x_L* and *bfl-1*

As shown in Fig. 3A, 4-1BB cross-linking increased transcription of two antiapoptotic genes, *bcl-x_L* and *bfl-1*, both of which have been shown to play critical roles in T lymphocyte survival. The

induction of *bcl-x_L* and *bfl-1* by 4-1BB was not blocked by pre-treatment of cells with CHX (Fig. 3B), indicating that 4-1BB-mediated signals increased the expression of *bcl-x_L* and *bfl-1* independently of new protein synthesis.

4-1BB-mediated up-regulation of *Bcl-x_L* and *Bfl-1* expression is blocked by PDTC, but not by LY294002 or PD98059

PDTC (20 μg/ml) completely inhibited 4-1BB-induced expression of *bfl-1* (Fig. 4, A and C) and *bcl-x_L* mRNA (Fig. 4, A and D), indicating that 4-1BB induces *bcl-x_L* and *bfl-1* mRNA expression via NF-κB. Similarly, 4-1BB-mediated increases in Bcl-x_L protein production were inhibited by PDTC (Fig. 4B). PDTC, an antioxidant compound, is known to specifically inhibit NF-κB via blockade of IκB-α phosphorylation (16). However, in contrast with its inhibition of 4-1BB-induced cellular proliferation (Fig. 1C), LY294002 had no effect on the 4-1BB-induced up-regulation of *bfl-1* (Fig. 5, A and C) or *bcl-x_L* mRNA (Fig. 5, A and D). Again, LY294002 did not inhibit the 4-1BB-mediated increase in Bcl-x_L protein production (Fig. 5B). Note that, although overall levels of Bcl-x_L and Bfl-1 expression were decreased by LY294002, the fold increase between lanes 1 and 2 was similar to that between lanes 3 and 4 (Fig. 5, A and B). These data suggest that 4-1BB-mediated induction of Bcl-x_L and Bfl-1 occurred via NF-κB activation, and not through the PI3K pathway.

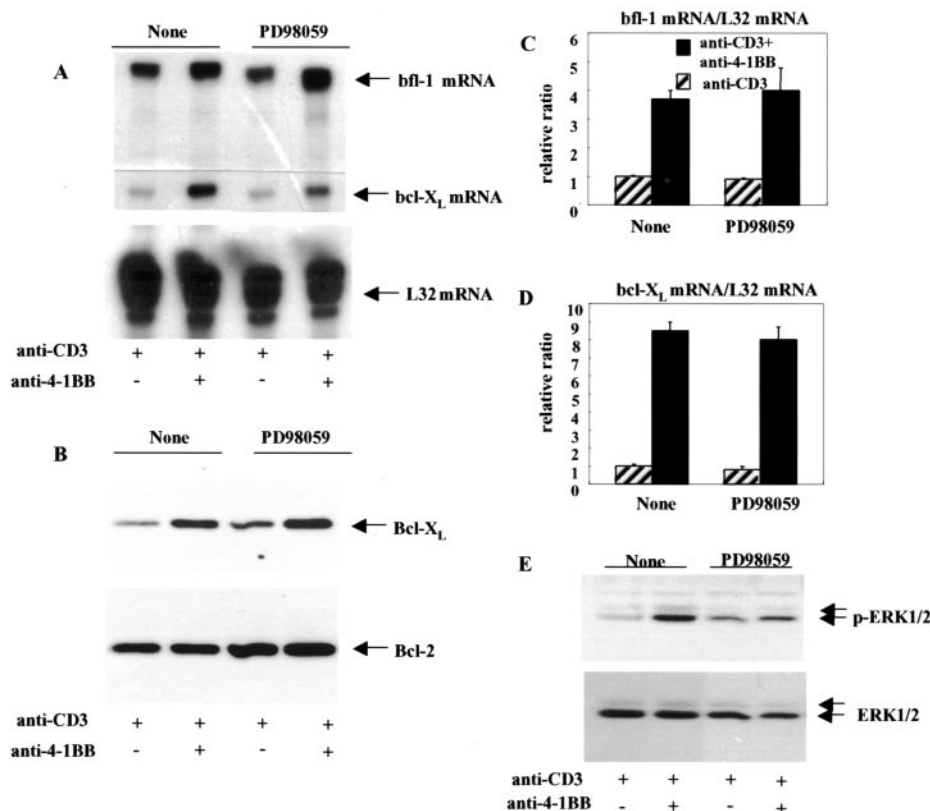


FIGURE 6. PD98059 does not block 4-1BB-induced expression of *bcl-x_L* and *bfl-1* mRNA (A) or Bcl-x_L protein (B). Anti-CD3 mAb-treated CD8⁺ T cells were preincubated with or without 30 μM PD98059 for 1 h, then with 5 μg/ml anti-4-1BB mAb or isotype control IgG. For RNase protection assay of *bcl-x_L* and *bfl-1* mRNA (A), total RNA was isolated after the cells were incubated with anti-4-1BB mAb or isotype control IgG for 6 h. Similar results were obtained in three independent experiments. For Western analysis of Bcl-x_L and Bcl-2 protein (B), proteins were extracted after a 24-h incubation with anti-4-1BB mAb or isotype control IgG. Similar results were obtained in three independent experiments. C, Densitometric measurements are presented as the relative abundance of *bfl-1* mRNA, compared with control L32 mRNA. D, Densitometric measurements are presented as the relative abundance of *bcl-x_L* mRNA, compared with control L32 mRNA. Values in C and D are means ± SD from three experiments. E, 4-1BB ligation with anti-4-1BB Ab increases phosphorylation of ERK1/2. Anti-CD3 mAb-treated CD8⁺ T cells were treated with 5 μg/ml anti-4-1BB mAb or isotype control IgG for 10 min in the presence or absence of 30 μM PD98059. Equal amounts of proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Phosphorylated ERK1/2 was detected by Western blotting using anti-phospho ERK1/2 Ab as the primary Ab (upper panel). After the membrane was stripped, ERK1/2 was detected by reprobng with anti-ERK1/2 Ab (lower panel). Similar results were obtained in two independent experiments.

In contrast, the MEK inhibitor PD98059, which blocked 4-1BB-mediated cellular proliferation (Fig. 1C), had no effect on 4-1BB-mediated increases in *bfl-1* (Fig. 6, A and C) or *bcl-x_L* mRNA (Fig. 6, A and D) or Bcl-x_L protein (Fig. 6B). Therefore, we determined whether 4-1BB would activate extracellular signal-related kinase 1/2 (ERK1/2), which is downstream of MEK. As shown in Fig. 6E, 4-1BB ligation increased phosphorylation of ERK1/2, but did not alter ERK1/2 protein levels. PD98059 inhibited 4-1BB-mediated-phosphorylation of ERK1/2 (Fig. 6E). Taken together, these data suggest that 4-1BB-mediated activation of the ERK1/2 and/or PI3K pathways is necessary for 4-1BB-induced proliferation, but not for 4-1BB-mediated Bcl-x_L or Bfl-1 expression or cell survival. Thus, it appears that 4-1BB-generated signals for cell survival are different from those that mediate cellular proliferation.

4-1BB-mediated NF- κ B activation is not coupled with the MEK or PI3K pathways

As shown in Fig. 7A, 4-1BB ligation resulted in a rapid degradation of I κ B- α that was evident within 5 min and persisted for up to 30 min. By 60 min, however, the levels of I κ B- α protein had increased sharply because of its rapid turnover rate (17). LY294002 and PD98059 had no effect on 4-1BB-mediated I κ B- α degradation, confirming that 4-1BB-mediated NF- κ B activation is not coupled with the PI3K or MEK pathways. However, I κ B- α degradation was inhibited by PDTC (Fig. 7B). NF- κ B activity was elevated in CD8⁺ T cells treated with anti-CD3 plus anti-4-1BB, relative to cells treated with anti-CD3 alone (Fig. 7C). In other studies (data not shown), we have seen that PDTC completely abrogates 4-1BB-induced NF- κ B activity.

Discussion

It has been shown previously that 4-1BB transmits a potent costimulatory signal to T cells, enhancing cell survival, promoting differentiation, and increasing cytokine expression (1). Recent in

vivo studies demonstrate that 4-1BB enhances the long-term survival of CD8⁺ T lymphocytes (2, 18). Here we present new findings on the 4-1BB signaling pathway specific for the survival of CD8⁺ T lymphocytes. First, we found that 4-1BB cross-linking by anti-4-1BB up-regulated expression of the anti-apoptotic genes *bcl-x_L* and *bfl-1* and increased production of Bcl-x_L protein. These effects appear to be responsible for 4-1BB-enhanced survival of primary CD8⁺ T lymphocytes. Second, we showed that, although 4-1BB-mediated ERK1/2 and/or PI3K signals enhanced proliferation of primary CD8⁺ T lymphocytes, these pathways were not involved in the 4-1BB-mediated increase in Bcl-x_L expression. Our data indicate that it is 4-1BB-mediated NF- κ B activation that provides CD8⁺ lymphocytes with prolonged survival via up-regulation of Bcl-x_L and Bfl-1 expression. It would be interesting to test whether inhibitors of *bcl-x_L* or *bfl-1* expression block 4-1BB-mediated antiapoptotic functions or whether 4-1BB-mediated costimulation is protective in *bcl-2*-deficient mice.

The critical importance of NF- κ B activation by 4-1BB for Bcl-x_L and Bfl-1 induction in our study is consistent with recent studies showing NF- κ B-dependent up-regulation of Bcl-x_L and Bfl-1 expression in other contexts (7–10). For instance, CD28-mediated NF- κ B activation is essential for Bcl-x_L induction and antiapoptotic effects in primary human CD4⁺ T lymphocytes (8). Similarly, NF- κ B-mediated up-regulation of Bcl-x_L and Bfl-1 is important for CD40 survival signaling in B lymphocytes (9). Although it has been shown that the PI3K/Akt pathway plays a role in NF- κ B activation (19, 20) and subsequent Bcl-x_L expression (10, 21), this pathway is not involved in 4-1BB-mediated up-regulation of Bcl-x_L and Bfl-1 in primary CD8⁺ T lymphocytes. LY294002, a PI3K blocker, abolished 4-1BB-mediated T cell proliferation to the same extent as did PDTC, an NF- κ B blocker. However, LY294002 did not block 4-1BB-mediated up-regulation of Bcl-x_L, whereas PDTC did. These data indicate that 4-1BB-induced PI3K and NF- κ B signals have separate physiological

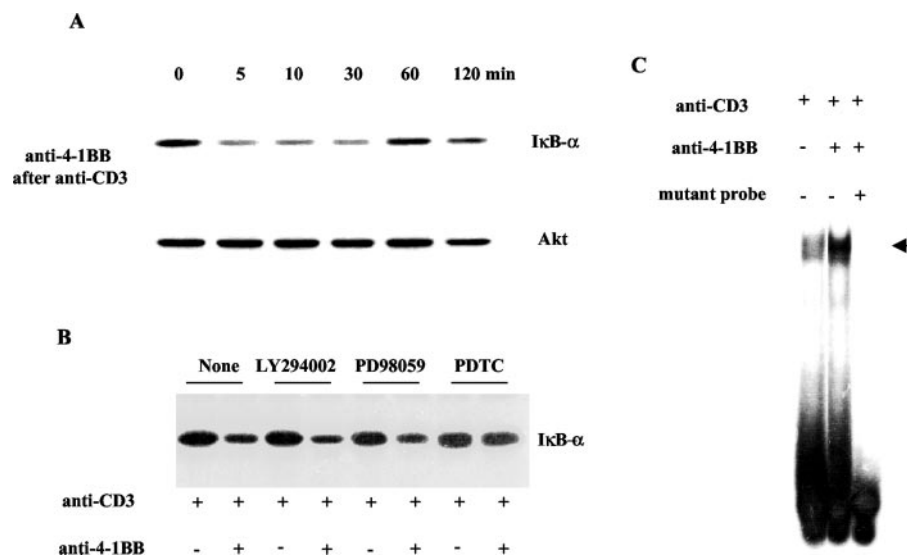


FIGURE 7. A, Treatment of CD8⁺ T cells with anti-4-1BB mAb induces degradation of I κ B- α . Anti-CD3 mAb-treated CD8⁺ T cells were treated with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for the indicated periods. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. I κ B- α was detected by Western blotting analysis with anti-I κ B- α Ab used as the primary Ab. After the membrane was stripped, Akt was detected by reprobing with anti-Akt Ab. Similar results were obtained in two independent experiments. B, 4-1BB-induced degradation of I κ B- α is not blocked by LY294002 or PD98059. Anti-CD3 mAb-treated CD8⁺ T cells were preincubated with vehicle, 30 μ M PD98059 or 20 μ M LY294002, for 1 h, then with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 10 min. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. I κ B- α was detected by Western blotting analysis using anti-I κ B- α Ab as the primary Ab. Similar results were obtained in two independent experiments. C, 4-1BB ligation enhances NF- κ B activity. Anti-CD3 mAb-treated CD8⁺ T cells were treated with 5 μ g/ml anti-4-1BB mAb or isotype control IgG for 1 h. EMSA analysis was conducted using a consensus NF- κ B binding element and its mutant element as described in *Materials and Methods*. Similar results were obtained in two independent experiments.

functions: only the NF- κ B signal triggers Bcl-x_L and, potentially, Bfl-1 expression. Interestingly, we have also observed that 4-1BB ligation enhances cell cycle progression and that PI3K and ERK1/2 signals are specifically responsible for 4-1BB-mediated cell cycle progression, but not for cell survival in CD8⁺ T lymphocytes (S.-J. Park, K.-O. Nam, B. S. Kwon, and H. W. Lee, manuscript in preparation). We speculate that 4-1BB-evoked cytokine secretion may act on cytokine receptors on CD8⁺ T cells in an autocrine or paracrine manner, thereby promoting cell cycle progression through the PI3K pathway. The IL-2R is well known to enhance cell cycle progression and proliferation via the PI3K/Akt/E2F/cyclin pathway (22, 23).

The present results explain how engagement of the costimulatory molecule 4-1BB enhances survival of CD8⁺ T lymphocytes. Our results show that 4-1BB-stimulated expression of Bcl-x_L and Bfl-1 occurs mainly through NF- κ B activation. This mechanism could account for 4-1BB-induced long-term survival of CD8⁺ T lymphocytes in vivo.

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