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4-1BB Promotes the Survival of CD8$^+$ T Lymphocytes by Increasing Expression of Bcl-x_L and Bfl-1[1]

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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 specificfor 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-pyrrollidinecarbodithioic 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 Biotechology.

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 10^8 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 10^6 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.

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

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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 μCi/well [^{3}H]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 μg/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 Na_3VO_4, 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× 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-μg 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 Na_3VO_4 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 Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/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 μl 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 μg of nuclear extract and ~0.5 ng of labeled oligonucleotides were incubated for 20 min at room temperature with 1 μg of poly(dI-dC) in 20 μl 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-κB binding element (top strand, AGTTGAGGGGACTTTCCCAGG) and its mutant element (top strand, AGTTGAGGCGACTTTCCCAGG) were labeled with [γ-^{32}P]ATP by means of polynucleotide kinase and used as probes for the κB 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 μg/ml anti-CD3 mAb (Fig. 1*A*). 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-κB inhibitor (PDTC; 20 μg/ml), a phosphatidylinositol-3 kinase (PI3K) inhibitor (LY294002; 20 μM), and two MAPK kinase (MEK) inhibitors (5 μM U0126 and 30 μM PD98059) (Fig. 1*C*). By contrast, a p38 MAPK inhibitor (SB203580; 20 μM) had no

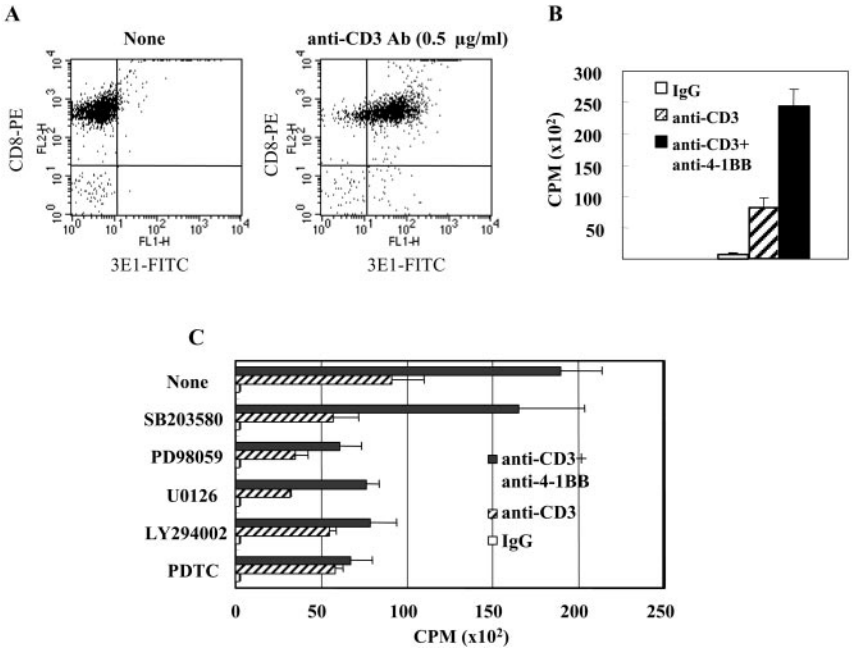

FIGURE 1. CD8$^+$ T lymphocytes (2×10^5-5×10^5) purified from lymph nodes and spleens of BALB/c mice using the MACS magnetic separation system were plated in a round-bottom 96-well microplate. The cells were incubated with 0.5 μg/ml anti-CD3 mAb or isotype control IgG for 16 h. *A*, 4-1BB expression 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 μg/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 μCi/well [^{3}H]thymidine. All cells were harvested and counted by liquid scintillation spectroscopy. The results are represented as means ± 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 μM SB203580, 30 μM PD98059, 5 μM U0126, 20 μM LY294002, or 20 μg/ml PDTC for 1 h and then with 5 μg/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 μCi/well [^{3}H]thymidine. All cells were harvested and counted by liquid scintillation spectroscopy. The results are represented as means ± SD of triplicates. Similar results were obtained in three independent experiments.

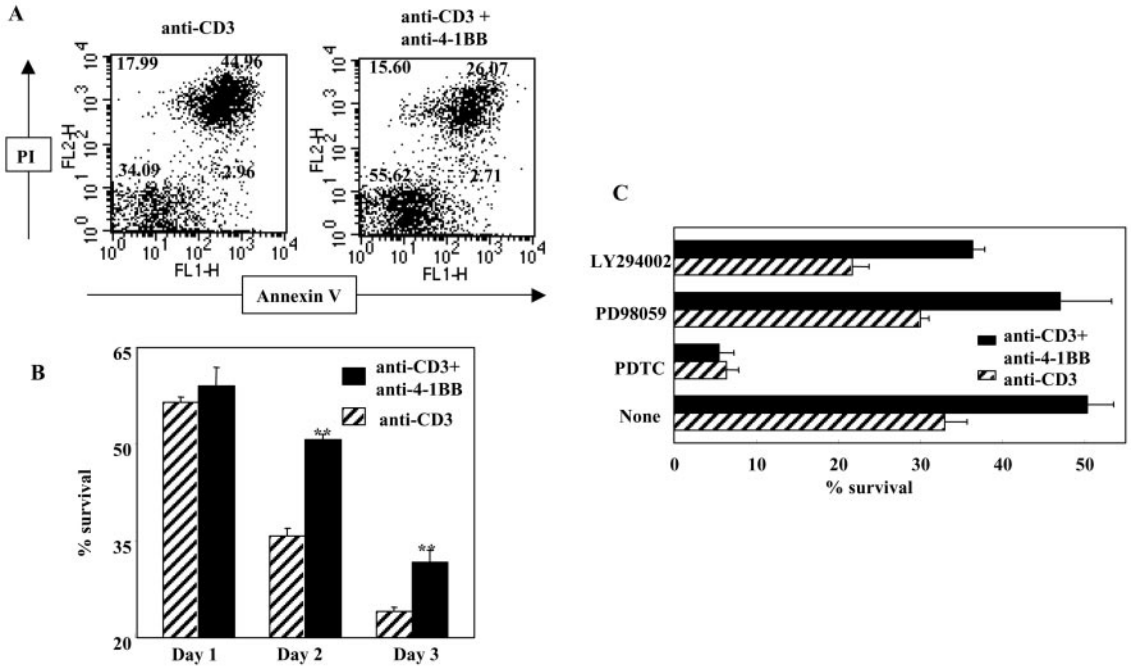

FIGURE 2. Treatment with anti-4-1BB mAb enhances survival of CD8$^+$ T cells. *A*, The percentage of cells surviving at various time points was quantitated by flow cytometric detection using gating of the double-negative portion of dual-color Annexin V-FITC/propidium iodide staining. FACS profiles on 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 ± 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 ± SD of triplicates. Similar results were obtained in two additional experiments.

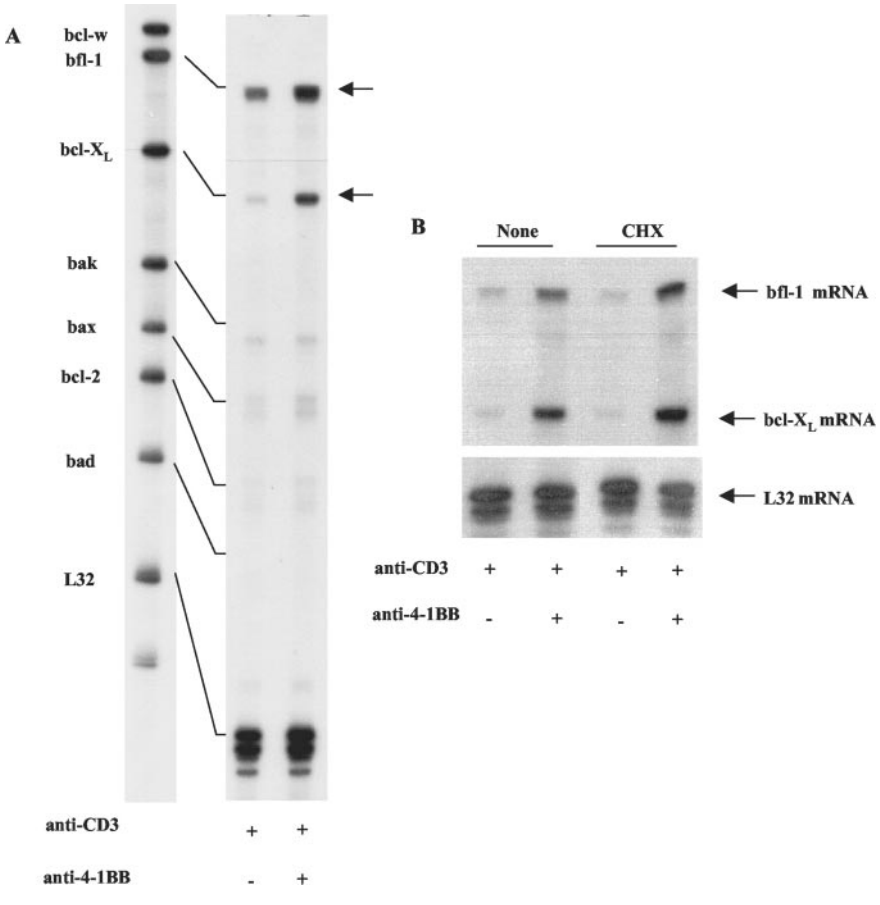

FIGURE 3. *A*, Treatment of CD8$^+$ T cells with anti-4-1BB mAb up-regulates expression of *bcl-x$_L$* and *bfl-1* mRNA. Anti-CD3 mAb-treated CD8$^+$ T cells were incubated with 5 μg/ml anti-4-1BB mAb or isotype control IgG for 6 h. The cells were lysed to extract total RNA. Equal amounts (9 μg) of total 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 μg/ml CHX for 1 h, then with 5 μg/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.

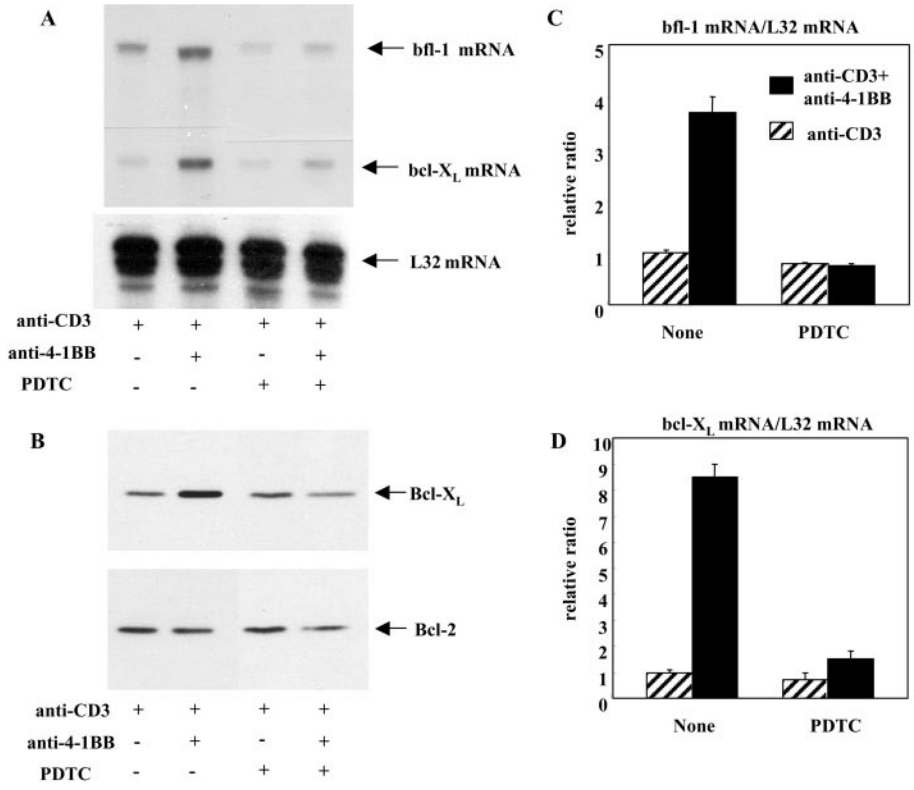

FIGURE 4. *A*, PDTC, an NF-κB inhibitor, blocks 4-1BB-induced up-regulation of *bcl-x$_L$* and *bfl-1* mRNA expression. *B*, PDTC inhibits 4-1BB-induced up-regulation of Bcl-x$_L$ protein, but it does not alter the level of Bcl-2 protein. Anti-CD3 mAb-treated CD8$^+$ T cells were preincubated with vehicle or 20 μg/ml PDTC (*A* and *B*) for 1 h, then with 5 μg/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 ± SD from three experiments.

effect (Fig. 1*C*). 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-κB, MEK, and PI3K signaling pathways 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

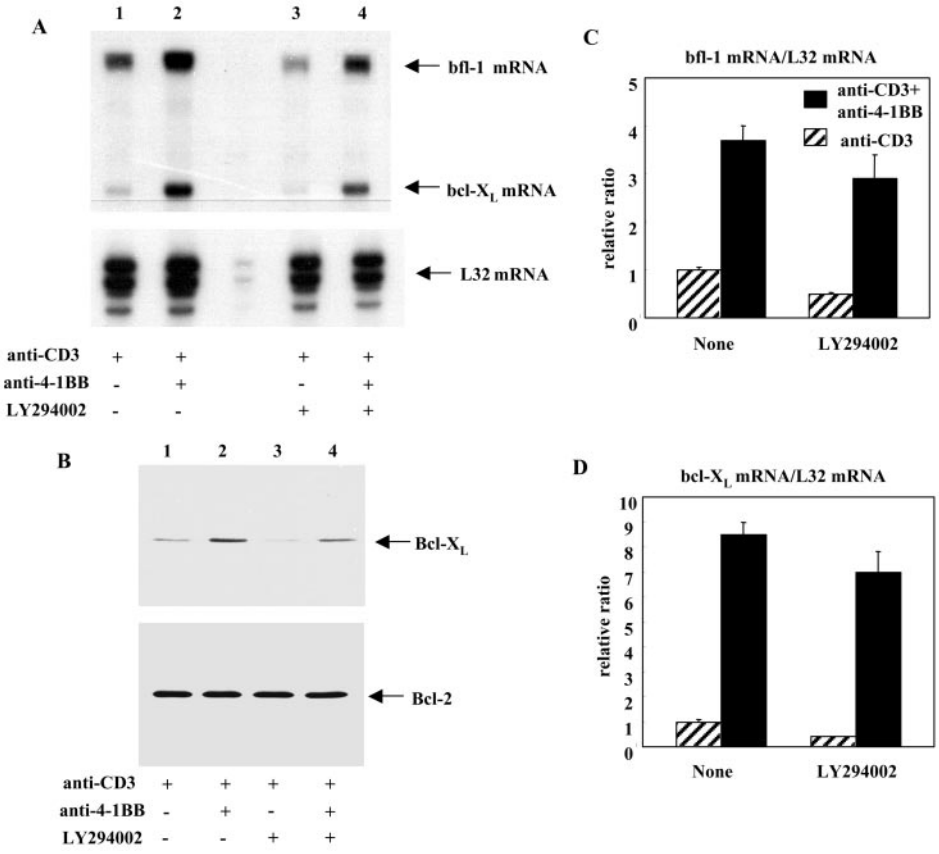

FIGURE 5. *A*, LY294002, a PI3K inhibitor, does not block 4-1BB-induced up-regulation of *bcl-x$_L$* and *bfl-1* mRNA expression. *B*, LY294002 does not block 4-1BB-induced up-regulation of Bcl-x$_L$ protein. Anti-CD3 mAb-treated CD8$^+$ T cells were preincubated with vehicle or 20 μM LY294002 (*A* and *B*) for 1 h, then with 5 μg/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 ± 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. 2*A* 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. 2*B*). 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. 2*C*). 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. 1*C*, whereas they had no effect on cell survival (Fig. 2*C*).

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

As shown in Fig. 3*A*, 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 pretreatment of cells with CHX (Fig. 3*B*), 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. 4*B*). 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. 1*C*), 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. 5*B*). 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.

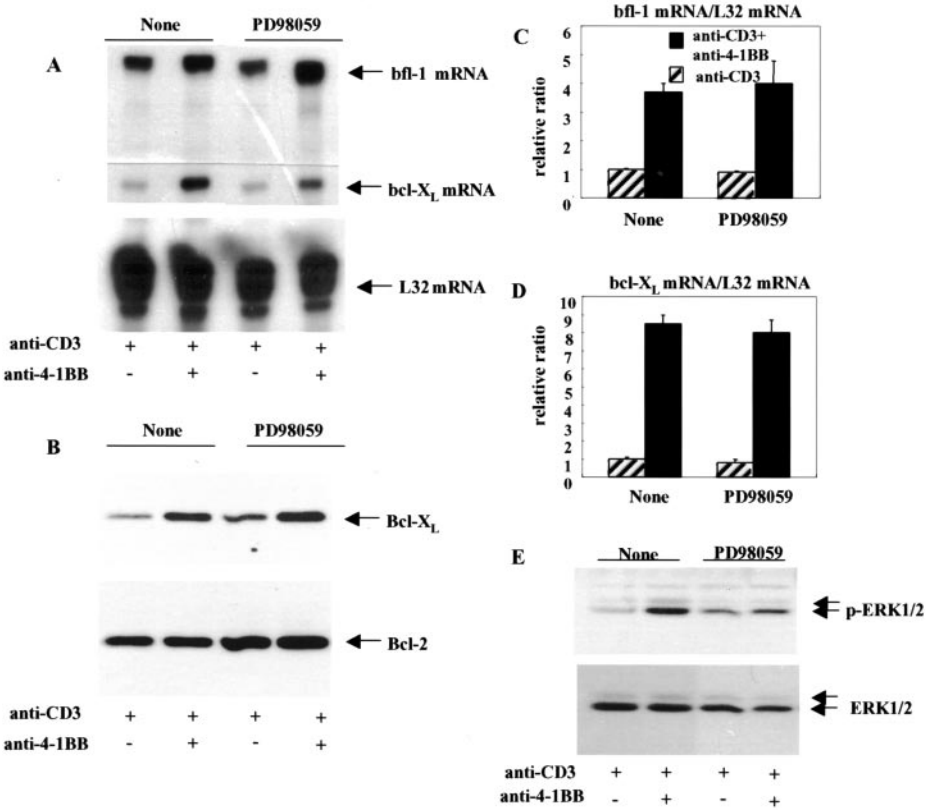

FIGURE 6. PD98059 does not block 4-1BB-induced expression of *bcl-x_L* and *bfl-1* mRNA (*A*) or Bcl-x_L protein (*B*). Anti-CD3 mAb-treated CD8$^+$ T cells were preincubated with or without 30 μM PD98059 for 1 h, then with 5 μg/ml anti-4-1BB mAb or isotype control IgG. For RNase protection assay of *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 reprobing 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. 1*C*), 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. 6*B*). 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. 6*E*, 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. 6*E*). 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-κB activation is not coupled with the MEK or PI3K pathways

As shown in Fig. 7*A*, 4-1BB ligation resulted in a rapid degradation of IκB-α that was evident within 5 min and persisted for up to 30 min. By 60 min, however, the levels of IκB-α protein had increased sharply because of its rapid turnover rate (17). LY294002 and PD98059 had no effect on 4-1BB-mediated IκB-α degradation, confirming that 4-1BB-mediated NF-κB activation is not coupled with the PI3K or MEK pathways. However, IκB-α degradation was inhibited by PDTC (Fig. 7*B*). NF-κB activity was elevated in $CD8^+$ T cells treated with anti-CD3 plus anti-4-1BB, relative to cells treated with anti-CD3 alone (Fig. 7*C*). In other studies (data not shown), we have seen that PDTC completely abrogates 4-1BB-induced NF-κB 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-κB 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-κB activation by 4-1BB for Bcl-x_L and Bfl-1 induction in our study is consistent with recent studies showing NF-κB-dependent up-regulation of Bcl-x_L and Bfl-1 expression in other contexts (7–10). For instance, CD28-mediated NF-κB activation is essential for Bcl-x_L induction and antiapoptotic effects in primary human $CD4^+$ T lymphocytes (8). Similarly, NF-κB-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-κB 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-κB 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-κB signals have separate physiological

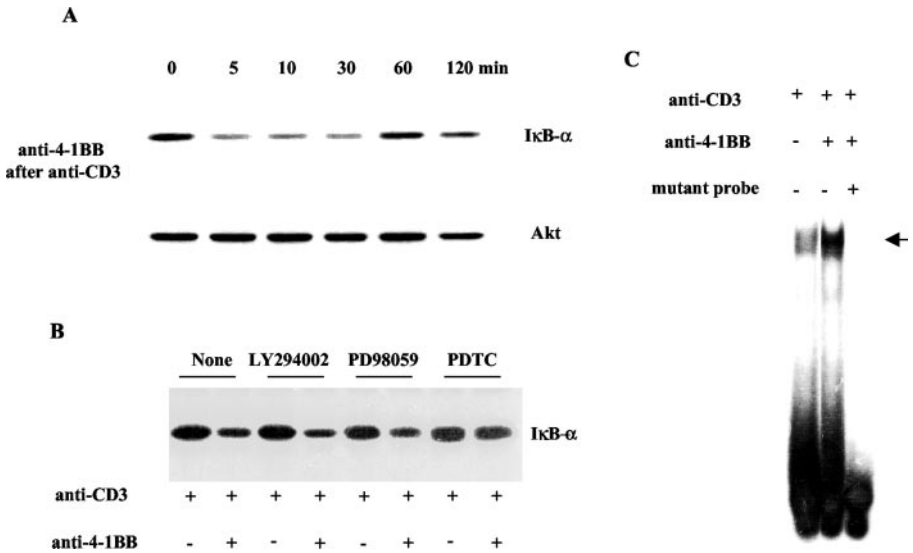

FIGURE 7. *A*, Treatment of $CD8^+$ T cells with anti-4-1BB mAb induces degradation of IκB-α. Anti-CD3 mAb-treated $CD8^+$ T cells were treated with 5 μg/ml anti-4-1BB mAb or isotype control IgG for the indicated periods. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose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

functions: only the NF-κB 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-κB activation. This mechanism could account for 4-1BB-induced long-term survival of CD8$^+$T lymphocytes in vivo.

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