CD28 Regulates the Translation of Bcl-xL via the Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Pathway

Linda X. Wu, Jose La Rose, Liane Chen, Chris Neale, Tak Mak, Klaus Okkenhaug, Ronald Wange and Robert Rottapel


http://www.jimmunol.org/content/174/1/180

---

**References**

This article cites 58 articles, 33 of which you can access for free at: http://www.jimmunol.org/content/174/1/180.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD28 Regulates the Translation of Bcl-xL via the Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Pathway

Linda X. Wu, Jose La Rose, Liane Chen, Chris Neale, Tak Mak, Klaus Okkenhaug, Ronald Wange, and Robert Rottapel

In concert with the TCR, CD28 promotes T cell survival by regulating the expression of the antiapoptotic protein Bcl-xL. The mechanism by which CD28 mediates the induction of Bcl-xL remains unknown. We show that although signaling through the TCR is sufficient to stimulate transcription of Bcl-xL mRNA, CD28, by activating PI3K and mammalian target of rapamycin, provides a critical signal that regulates the translation of Bcl-xL transcripts. We observe that CD28 induced 4E-binding protein-1 phosphorylation, an inhibitor of the translational machinery, and that CD28 costimulation directly augmented the translation of a Bcl-xL 5′-untranslated region reporter construct. Lastly, costimulation by CD28 shifted the distribution of Bcl-xL mRNA transcripts from the pretranslation complex to the translationally active polyribosomes. These results demonstrate that CD28 relieves the translational inhibition of Bcl-xL in a PI3K/mammalian target of rapamycin-dependent manner. The Journal of Immunology, 2005, 174: 180–194.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication February 9, 2004. Accepted for publication October 15, 2004.

The Journal of Immunology

Copyright © 2005 by The American Association of Immunologists, Inc.
these proteins are poorly translated in naive and resting cells, although their rate of translation is potently induced after growth stimulation (21, 28).

In this study we show that CD28 regulates Bcl-xL protein expression, but not the steady-state level of mRNA. Both LY294002 and rapamycin abrogated CD28-induced Bcl-xL protein production without affecting mRNA expression. CD28 costimulation induced phosphorylation of 4E-BP1, an effect that was inhibited by both LY294002 and rapamycin. CD28 costimulation also relieved the translational inhibition via the 5′-untranslated region (5′UTR) of Bcl-xL in a luciferase reporter gene system. CD28 significantly increased the binding of Bcl-xL mRNA to polyribosomes, thereby augmenting the translational efficiency of Bcl-xL transcripts. These findings show that CD28 couples to the cap-dependent translational machinery through which it regulates the expression of proteins, such as Bcl-xL, that are required for propagation of the immune response.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. CD28−/− mice were backcrossed to the C57BL/6 background for >10 generations. Wild-type CD28 transgenic mice (WT Tg) and mutant CD28 transgenic mice (Y170F) were described previously (12). Gag-PKB mice (29) were a gift from Dr. J. Woodgett (University of Toronto, Toronto, Canada). Mice were used at 8–20 wk of age and were age-matched within 4 wk in any given experiment. Mice were housed in a specific pathogen-free facility, and their derivation and use were in compliance with the animal care committee guidelines of the Ontario Cancer Institute (Toronto, Canada).

Abs and reagents

The murine CD28-specific mAb, 37.51, PE-anti-Thy1.2 (53-2.1), and PE-anti-Bcl-2 mAb were purchased from BD Pharmingen. The murine CD3-specific mAb 145-2C11 was purified from hybridoma supernatant with protein A chromatography. Anti-human CD28 mAb 9.3 ascites was a gift from Dr. P. Linsley (Bristol-Meyers Squibb). FITC-anti-Bcl-xL (7B2.5) was purchased from Southern Biotechnology Associates. Phospho-4E-BP1 (Ser473), Abs that detect Ser37/46 or Thr37/46 phosphorylated-4E-BP1 were purchased from Cell Signaling Technology. Anti-4E-BP1 Ab and pACTAG-2 vectors containing WT 4E-BP1 or BP1-Y37F were gifts from Dr. N. Sonenberg (McGill University, Montréal, Canada). Anti-Bcl-xL Ab from Santa Cruz Biotechnology. The blocking anti-murine IL-2 Ab, S4B6-1, was a gift from Dr. R. Miller (University of Toronto). Anti-β-actin mAb (AC-15), rapamycin, cycloheximide, PMA, and ionomycin were purchased from Sigma-Aldrich. LY294002 was purchased from Calbiochem. pGL3-Luciferase vector was purchased from Promega. The PTEN WT tet-on and PTEN G129R vectors were obtained from Dr. R. Wange (National Institutes of Health, Bethesda, MD). Anti-PTEN Ab was a gift from Dr. V. Stambolic (University of Toronto).

Activation-induced cell death (AICD)

Peripheral T lymphocytes were cultured from spleen or lymph nodes in a complete medium (α-MEM; 10% FCS, 50 mM 2-ME, 2 mM l-glutamine, and 100 mM HEPES). Splenocytes or lymph node cells were cultured at 2 × 106 cells/ml with 1 μg/ml soluble anti-CD3 for 48 h in the presence of 20 U/ml mouse IL-2 or the equivalent amount of human IL-2 (Proleukin; Ligand Pharmaceuticals) as titrated on the IL-2-dependent CTLL-2 cell line. Activated T cell blasts were isolated using Lymphoprep density separation medium (Cedarlane Laboratories) and re-cross-linked at a concentration of 5 × 106 cells/ml in the presence of IL-2 on 96-well, high binding plates (Corning Costar) coated with 1 μg/ml anti-CD3 and blocked with 10% FCS in PBS. Controls that were not re-cross-linked were maintained with IL-2 and plated on FCS-coated wells.

Cell culture

Lymph node cells from C57BL/6, CD28−/−, WT Tg, and Y170F mice were cultured at 2 × 106 cells/ml and stimulated with anti-CD3 and anti-CD28 Abs (1 μg/ml) or PMA (10 ng/ml) plus ionomycin (100 ng/ml) in the presence or the absence of the blocking anti-IL-2 Ab (S4B6-1; 50 μg/ml). Cells were cultured in 24-well plates for 18 h at 37°C. For the RT-PCR experiments, lymph node cells either were left unstimulated or were stimulated with anti-CD3 and anti-CD28 Abs (1 μg/ml) in the presence of 10 μM LY294002 or 10 nM rapamycin. For the dose-response studies of inhibitors, lymph node cells from C57BL/6 either were left unstimulated or were stimulated with anti-CD3 Ab (1 μg/ml) and/or anti-CD28 Ab (1 μg/ml) in the presence or the absence of rapamycin (ranging from 1 μM to 0.1 nM) or LY294002 (ranging from 100 μM to 100 nM).

Flow cytometry

The cells were harvested after 18 h of culture, washed once with PBS, stained with PE-Thy1.2 (1 μg/ml) or H57-FITC (1 μg/ml), fixed at room temperature with 1 ml of 3.7% formaldehyde in PBS for 2 min, and permeabilized at room temperature for 10 min with 0.15% Triton X-100. The cells were then washed once with PBS and stained with FITC-Bcl-xL (1 μg/ml) or PE-Bcl-2 (1 μg/ml). For hypodiploid DNA analysis, T cells were fixed on ice in a final ethanol concentration of 50% and stored at 4°C. Cells were washed with Ca2+/Mg2+-free PBS and resuspended in staining buffer (5 μg/ml 7-aminomethylenzo (7AAD)/PBS) at room temperature for 15 min. Cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences).

RT-PCR

Cells (8 × 106) were harvested and lysed with an RNeasy Mini kit buffer RLT (Qiagen). The lysates were homogenized with a QIA shredder column (Qiagen). Total RNA was extracted using the RNeasy Mini kit (Qiagen) and was reverse transcribed using the Thermoscript RT-PCR system (Invitrogen Life Technologies). cDNA was then serially diluted in dH2O before being subjected to PCR with Platinum Taq DNA polymerase (Invitrogen Life Technologies) to detect the presence of Bcl-xL and GAPDH. The following primers were used to amplify specific genes as follows: Bcl-xL 5′-TGGCTGGCCACATCCCGCTACATACACCC-3′ and 5′-CCACAAAGACAGCCTGAC-3′; GAPDH 5′-TGTCATGTCGAAGAAGGATGGAGAT-3′ and 3′-TCTCCATTCTCGGCGTTGA-3′.

Jurkat tet-on and Jurkat PTEN WT tet-on were transfected with pG3L (Promega) or pG3L Bcl-xL 5′-UTR using the FuGene 6 transfection reagent (Roche). After 24 h, transfected cells were treated with or without 1 μg/ml doxycyclin (Sigma-Aldrich Canada). Cells were cultured for an additional 24 h. Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (BD Biosciences), 2 mM glutamine, 50 mM 2-ME, 10 mM HEPES, penicillin, and streptomycin. Total RNA was extracted using the RNeasy Mini kit (Qiagen). mRNA was prepared from the total RNA using the oligotex mRNA Mini kit (Qiagen). One hundred nanograms of mRNA was reversed transcribed into cDNA using the Thermoscript RT-PCR system (Invitrogen Life Technologies). PCR were performed with Platinum Taq DNA polymerase (Invitrogen Life Technologies) using primers to detect luciferase and GAPDH. Primer sequences are: luciferase 5′-TCAGGAACGCGGCAACAGTTG-3′ and 3′-GGTGTTGGAGCAAGAGTGAT-3′; GAPDH 5′-CAACTCCTGAAAGTGATAT-3′ and 3′-GCCTGCGTCGTTGTAGC-3′.

Quantitative real-time PCR

DNA was extracted and quantitated. Two micrograms of DNA was reverse transcribed to produce cDNA using the Thermoscript RT-PCR system (Invitrogen Life Technologies). Real-time PCR was performed in a final volume of 25 μl containing SYBR Green PCR master mix (Applied Biosystems), primers, and cDNA. The following primers were used to amplify specific genes as follows: Bcl-xL 5′-TACCGAGAGGCGTTCAGTGA-3′ and 3′-CCAGTTTACTCCATCTCC-3′; GAPDH 5′-CCAGTTTACTCCATCTCC-3′ and 3′-CCAGTTTACCAACGCTG-3′.

Immunoblotting and immunoprecipitation

Cells were washed twice with PBS and lysed in phosphatase Cy lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, and 1 mM EGTA, pH 7) on ice for 1 h. The lysates were resolved by SDS-PAGE using 10% polyacrylamide gels, electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and

Downloaded from http://www.jimmunol.org/ by guest on July 25, 2017
probed with Bcl-xL-specific Ab at a 1/200 dilution (Santa Cruz Biotechnology) and protein A-HPRT at a 1/25,000 dilution. The blot was stripped and reprobed with an actin-specific Ab at a 1/1,000 dilution (AC-15; Sigma-Aldrich) and anti-mouse-HPRT at a 1/1,000 dilution as the secondary probe to ensure equal loading of protein. A similar procedure was used in immunoprecipitation to analyze the phosphorylation of 4E-BP1. Lymph node cells were stimulated with anti-CD3 and/or anti-CD28 (1 µg/ml) in the presence or the absence of rapamycin (10 nM) for 18 h. Cells were then harvested and lysed in phospholipase C buffer. The detergent-insoluble fraction was removed by centrifugation, and the supernatants were incubated with protein A-Sepharose beads (10 µg) and 10 µg of anti-4E-BP1 at 4°C for 1 h. The beads were washed with lysis buffer and then boiled in 2× Laemmli-SDS buffer (2% SDS, 100 mM Tris-HCl, pH 6.8, 20% 2-ME, and 0.01% bromophenol blue). The proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blotted with anti-p-4E-BP1 at a 1/1,000 dilution. Strips were reprobed with anti-4E-BP1 (1/200 dilution) and anti-actin (1/1,000 dilution), with anti-rabbit HRP (1/7,000 dilution) as the secondary blotting Ab, respectively. Proteins were revealed by Enhanced Luminol reagent (DuPont, NEN) upon exposition to film.

Transfection of Jurkat T Cells and luciferase assay

Jurkat T cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, penicillin, and streptomycin. Jurkat cells in the logarithmic growth phase were transfected by electroporation. Cells (2 × 10⁷) were resuspended in 400 µl of serum-free RPMI 1640. Plasmid DNA was mixed with the cells in a 4-mm gap electroporation cuvette and pulsed at 250 V and 960 µF using the Gene Pulser (Bio-Rad). The cells were then transferred to culture flasks and incubated in complete medium. Jurkat T cells were cultured at 37°C for 18 h before anti-CD3 (OKT3) and/or anti-CD28 (9.3) Abs were added at 10 µg/ml for an additional 6 h. Cells were harvested, washed once in PBS, and lysed in Reporter lysis 1 (H11003) buffer (Promega). Luciferase activity was measured on a LUMAT LB 9507 luminometer (EG&G Berthold Systems).

Quantitation of IL-2 produced by lymph node cells

Lymph node cells from C57BL/6 mice were cultured at 2 × 10⁶ cells/ml and stimulated with or without anti-CD3 and anti-CD28 Abs (1 µg/ml). Cells were cultured in 24-well plates in 1 ml of RPMI 1640, 10% FCS, 2 mM glutamine, 50 mM 2-ME, 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin for 48 h at 37°C in 5% CO2. Cells were pelleted, washed twice in PBS, and resuspended in 100 µl of serum-free RPMI 1640 containing 10% FCS, 50 mM 2-ME, and 10 mM HEPES. Cells were cultured in 24-well plates in 1 ml of RPMI 1640, 10% FCS, 50 mM 2-ME, and 10 mM HEPES. The proliferative response was determined by IL-2 ELISA kit (R&D Systems).

CTLL-2 assay

CTLL-2 cells were grown in 96-well plates at a concentration of 5 × 10³ CTLL-2 cells/well in 200 µl of RPMI 1640, 10% FCS, 50 mM 2-ME, and 10 mM HEPES. Cells were stimulated with 110 U/ml recombinant mouse IL-2 (R&D Systems) in the presence of the blocking anti-IL-2 Ab (54B6-1; from R. Miller’s laboratory, Ontario Cancer Institute, Toronto, Ontario, Canada) in 200 µl of RPMI 1640, 10% FCS, 50 mM 2-ME, and 10 mM HEPES. The proliferative response was determined by [3H]thymidine incorporation at 48 h.

Results

Prevention of AICD by CD28

CD28 promotes T cell survival in part through the up-regulation of Bcl-xL (7). Mice harboring a tyrosine to phenylalanine mutant of CD28 (Y170F) no longer recruit SH2-containing signaling proteins such as PI3K, fail to up-regulate Bcl-xL, and are sensitive to stress-induced apoptosis (12). T cells undergo apoptosis when the TCR/CD3 complex is re-cross-linked in the absence of costimulation during a primary immune response, in a process known as AICD. Prevention of AICD by CD28 is associated with increased expression of Bcl-xL, but not Bcl-2. We examined the physiological role of Y170 in CD28-mediated up-regulation of Bcl-xL and subsequent prevention of AICD by measuring the frequency of hypodiploid cells (Fig. 4a) or by annexin V/7AAD staining using flow cytometry (data not shown). We measured AICD in C57BL/6- and WT Tg-derived splenocytes by determining the percent increase in hypodiploid cells after CD3 reiation (10 to 15% and 7 to 20%, respectively). Similarly, we observed a significant increase in the frequency of hypodiploid cells in CD28−/− (12 to 21%), and in the Y170F mutant splenocytes (13 to 24%) undergoing AICD. CD3/CD28 costimulation reduced the percentage of hypodiploid cells from 15 to 10% in C57BL/6-derived splenocytes and from 20 to 10% in WT Tg-derived splenocytes, comparable to the non-re-cross-linked T cell controls growing in IL-2. The CD28-mediated protective effect against AICD was not observed in CD28−/− mice.
CD28 prevents early AICD. 

a. Splenocytes from C57BL/6, CD28−/−, WT Tg, and Y170F mice were stimulated with soluble anti-CD3 Ab in the presence of IL-2 for 48 h, and purified T cells were re-cross-linked with plate-bound anti-CD3 or anti-CD3 and anti-CD28 Abs for an additional 24 h. Non-re-cross-linked controls were maintained in IL-2. Cells were stained with 7AAD, and DNA content was analyzed by FACS. 

b. Statistical analysis of the percentage of hypodiploid cells from data averaged over five individual experiments with SEs. □, Non-re-cross-linked control cells; ■, cells re-cross-linked with anti-CD3 Ab; ○, cells re-cross-linked with anti-CD3 and anti-CD28 Abs.
CD28 induction of Bcl-xL is independent of IL-2, and the CD28 pathway does not affect Bcl-2 protein expression. a. Lymph node cells from C57BL/6 were stimulated with anti-CD3/CD28 Abs. Unstimulated controls were maintained in medium. Cells were stained (Figure legend continues)

FIGURE 2. CD28 induction of Bcl-xL is independent of IL-2, and the CD28 pathway does not affect Bcl-2 protein expression. a. Lymph node cells from C57BL/6 were stimulated with anti-CD3/CD28 Abs. Unstimulated controls were maintained in medium. Cells were stained (Figure legend continues)
FIGURE 3. Bcl-xL mRNA expression is dependent on CD3 ligation, but independent of CD28 costimulation. 

a. Lymph node cells from C57BL/6 and CD28−/− were unstimulated or stimulated with anti-CD3 and/or anti-CD28 Abs. Total RNA was subjected to RT-PCR to generate cDNA. Total cDNA was then analyzed by quantitative real-time PCR for Bcl-xL and GAPDH gene expression. Experiments were conducted in triplicate. Real-time PCR data are represented as relative expression units of Bcl-xL to GAPDH.

b. Lymph node cells from C57BL/6 and CD28−/− were unstimulated or stimulated with anti-CD3 Ab and/or anti-CD28 Ab in the absence or the presence of LY294002 (10 μM) or rapamycin (10 nM) for 18 h. Total RNA was subjected to RT-PCR to synthesize cDNA. Serial dilutions (1/1, 1/5, 1/25, and 1/125) of cDNAs were used in the PCR with primers specific for Bcl-xL and GAPDH to give a respective representation of the steady-state level of the mRNA expression.
observed in CD28-deficient T cells or in those harboring the CD28 Y170F mutant, with respective hypodiploid cell percentages remaining at 21 and 25% after CD3 re-ligation (Fig. 1a). These data averaged over five individual experiments are shown in Fig. 1b. A test comparison demonstrated a strong statistical difference between the protective effect of wild-type CD28 compared with CD28-deficient T cells (p < 0.001). The Y170F mutant afforded weak, but measurable, protection from early AICD in comparison with CD28−/− T cells (p < 0.01). These results confirm that CD28 costimulation confers protection of T cells from early AICD and demonstrate that this survival signal is coupled to residue Y170 of CD28.

CD28-induced Bcl-xL protein expression is independent of IL-2 production and is specific for Bcl-xL, but not Bcl-2

CD28 together with CD3 cross-linking increases Bcl-xL protein levels, as determined by intracellular staining flow cytometry (Fig. 2a).

IL-2 can also activate PI3K (33, 34) and up-regulate Bcl-xL (35). To determine whether the induction of Bcl-xL protein expression was a secondary response of CD28-dependent IL-2 production, Bcl-xL expression was measured under conditions where IL-2 activity was neutralized by the anti-IL-2 mAb, S4B6–1. IL-2 production after CD3/CD28 costimulation was 110 U/ml, as measured by ELISA in our T cell culture system (Fig. 2a). An inhibition assay was then set up using 110 U/ml rIL-2 in a CTLL-2 proliferation assay in the presence of increasing doses of S4B6–1 (Fig. 2b). CTLL-2 proliferation was inhibited > 95% with 10 μg/ml S4B6–1. We then tested the capacity of CD3/CD28 costimulation to induce Bcl-xL protein in the presence of a 5-fold excess of Ab (50 μg/ml) and observed no attenuation in Bcl-xL compared with the isotype control (Fig. 2c). These data show that CD28 regulates Bcl-xL protein expression independently of IL-2 activity.

Bcl-2 is another antiapoptotic protein that shares high sequence homology with Bcl-xL. FACS analysis revealed that Bcl-2 was...
constitutively expressed in murine primary T cells, and its expression level was not altered by CD28 costimulation (Fig. 2d). In addition, the constitutive expression level of Bcl-2 was not affected by the presence of LY294002, a PI3K inhibitor, or rapamycin, an mTOR inhibitor (Fig. 2e). Therefore, CD28 dynamically regulates Bcl-x<sub>L</sub> protein expression, but not Bcl-2.

**Regulation of Bcl-x<sub>L</sub> mRNA expression**

Protein expression is controlled at multiple levels by the regulation of transcription, mRNA stability, translation, and protein degradation. To determine whether CD28 regulates Bcl-x<sub>L</sub> expression at the mRNA level, lymph node cells harvested from C57BL/6 and CD28<sup>−/−</sup> mice were stimulated with anti-CD3 and/or anti-CD28 Abs. RNA was then extracted, and the expression level of Bcl-x<sub>L</sub> mRNA was quantified by real-time RT-PCR analysis. Resting T lymphocytes expressed low basal levels of Bcl-x<sub>L</sub> transcript. Stimulation with anti-CD3 Ab alone was sufficient to potently induce Bcl-x<sub>L</sub> mRNA, whereas there was no further enhancement of Bcl-x<sub>L</sub> mRNA levels after costimulation with anti-CD3 and anti-CD28 Abs (Fig. 3a). Lymph node cells derived from CD28<sup>−/−</sup> mice were capable of up-regulating Bcl-x<sub>L</sub> mRNA in response to anti-CD3 stimulation at a level comparable to that observed in C57BL/6-derived lymphocytes (Fig. 3a). Similar results were observed using semiquantitative RT-PCR analysis (Fig. 3b). Stimulation with anti-CD28 alone did not induce Bcl-x<sub>L</sub> mRNA expression, whereas CD3, independently of CD28, was capable of maximally increasing Bcl-x<sub>L</sub> transcript. Neither LY294002 nor rapamycin inhibited Bcl-x<sub>L</sub> mRNA expression levels (Fig. 3c), showing that neither PI3K nor mTOR was required for steady state control of Bcl-x<sub>L</sub> mRNA. These data show that CD3, but not CD28, is sufficient for the induction of Bcl-x<sub>L</sub> transcripts, suggesting that CD28 probably regulates Bcl-x<sub>L</sub> protein expression at the translational or post-translational level.

**Effects of PI3K and mTOR inhibitors on Bcl-x<sub>L</sub> protein expression**

To investigate whether the PI3K pathway is involved in CD28-dependent regulation of Bcl-x<sub>L</sub> at the protein expression level, we treated lymph node cells with LY294002 for 18 h. CD28-dependent induction of Bcl-x<sub>L</sub> protein expression was inhibited by LY294002 in a dose-dependent fashion, with significant inhibition observed at 10 μM (Fig. 4, a and b). We next used rapamycin, to examine whether mTOR was involved in PI3K-dependent CD28 induction of Bcl-x<sub>L</sub> protein expression. Rapamycin exhibited a dose-dependent inhibition of Bcl-x<sub>L</sub> protein expression, with significant inhibition observed at 10 nM (Fig. 4, c and d). These data show that the inhibition of either PI3K or mTOR activity reduces CD28-induced Bcl-x<sub>L</sub> protein expression.

**Role of CD28 in 4E-BP1 phosphorylation**

Our data suggest that CD28 regulates Bcl-x<sub>L</sub> protein expression through the PI3K/PKB/mTOR-dependent pathway. We next examined whether 4E-BP1, a major downstream target of mTOR (36), is involved in translational regulation of Bcl-x<sub>L</sub>. 4E-BP1 is the inhibitory binding partner of eIF4E and is unphosphorylated in resting T cells. Phosphorylation of 4E-BP1 releases eIF4E and allows the recruitment and assembly of the ribosomal translational machinery at the cap region. Stimulation with CD3/CD28, but not CD3 alone, markedly enhanced 4E-BP1 phosphorylation at both Ser<sup>65</sup> (Fig. 5a) and Thr<sup>70</sup> (data not shown) in purified C57BL/6 T cells. Similarly, no 4E-BP-1 phosphorylation was observed after cross-linking of CD3 in CD28-deficient T cells. Treatment of purified T cells with LY294002 or rapamycin before costimulation completely blocked 4E-BP1 phosphorylation, demonstrating the requirement for PI3K and mTOR in CD28-induced 4E-BP1 phosphorylation (Fig. 5b).

To determine whether the activation of PKB is sufficient to trigger a pathway leading to 4E-BP1 phosphorylation, we examined the phosphorylation state of 4E-BP1 in T cells derived from Gag-PKB Tg mice, where PKB is constitutively active (29, 37). We observed that 4E-BP1 was phosphorylated in naive T cells derived from Gag-PKB Tg mice, and that the 4E-BP1 phosphorylation was significantly enhanced by CD28 costimulation, suggesting that PKB is an intermediary between CD28 and 4E-BP1 (Fig. 5c).
CD28 REGULATES Bcl-x<sub>L</sub> TRANSLATION

FIGURE 6. CD28 costimulation induces translation of Bcl-x<sub>L</sub>. (a) Jurkat T cells were transfected in triplicate with pGL3–5'UTR Bcl-x<sub>L</sub>-luciferase or pGL3-luciferase vectors by electroporation. After 18 h, cells stimulated with anti-CD3 (OKT3; 10 μg/ml) and/or anti-CD28 (9.3; 10 μg/ml) for an additional 6 h, *p < 0.01. (b) Jurkat T cells were cotransfected in triplicate with pGL3–5'UTR Bcl-x<sub>L</sub>-luciferase and pACTAG2–4E-BP1 WT or pACTAG2-BP1-D4E plasmids by electroporation for 18 h, then stimulated with anti-CD3 and anti-CD28 Abs for an (Figure legend continues)
**CD28 relieves translational inhibition of 5′ UTR of Bcl-xL in a 4E-BP1-dependent manner**

To determine whether CD28 costimulation directly regulates translation of Bcl-xL, we cloned and inserted the 5′ UTR (nt −447-1) of Bcl-xL into a luciferase reporter plasmid and transfected it into Jurkat T cells. Upon costimulation with CD3/CD28, transfected Jurkat T cells showed a significant increase in luciferase activity ($p < 0.01$) compared with cells stimulated with CD3 or CD28 alone (Fig. 6a). This effect was completely repressed by cotransfection with 4E-BP1 WT vector (Fig. 6b). Overexpression of 4E-BP1 favors the accumulation of unphosphorylated 4E-BP1, which inhibits the cap-dependent translation. Cotransfection of a mutant form of 4E-BP1, BP1-D4E, deficient in its capacity to sequester and inhibit eIF-4E, together with the pGL3–5′UTR Bcl-xL luciferase vector did not repress luciferase reporter activity (Fig. 6b), indicating that 4E-BP1 plays a specific inhibitory role in translational regulation of Bcl-xL. Jurkat T cells transfected with empty luciferase vector did not exhibit differential luciferase activity under different stimulation conditions (Fig. 6a).

Jurkat T cells lack the PIP3 phosphatase PTEN and have a high steady state level of PIP3 (38). To test for the requirement for PTEN in the CD28-dependent regulation of Bcl-xL translation, we used a PTEN tet-on system in Jurkat T cells. We observed that Jurkat cells grown in the presence of doxycyclin expressed PTEN (Fig. 6d) and were still viable, but grew more slowly (data not shown). Overexpression of PTEN completely repressed the ambient level of Bcl-xL-luciferase translation, an effect that could not be overcome by CD28-dependent costimulation (Fig. 6c). This repression was alleviated and, in fact, augmented by cotransfection of an inactive form of PTEN harboring a mutation in its active site (PTEN G129R). Both PTEN WT and PTEN G129R were induced at comparable levels with 1 μg/ml doxycyclin at 24 h (Fig. 6d). Induction of PTEN in Jurkat cells also shut down the basal level of luciferase translation in cells expressing the pGL3-luciferase control plasmid (Fig. 6e). To exclude the possibility that PTEN expression interfered with transcription of the luciferase reporter constructs, semiquantitative RT-PCR was used to examine luciferase mRNA expression when the pGL3-3 control and Bcl-xL constructs were introduced in both parental Jurkat T cells and PTEN WT Jurkat T cells (Fig. 6f). We observed that PTEN had no effect on Bcl-xL-luciferase mRNA expression levels. These data show that inhibition of the PI3K pathway by inducible expression of its inactive form PTEN G129R, whereas the translationally active mRNA fraction is enriched in fractions 11–20 (39). The distribution of Bcl-xL mRNA transcripts associated with each pool was detected by Northern blot analysis after T cell activation with CD3 and CD28 from C57BL/6, CD28−/− or Y170F T cells. Cytoplasmic extracts from stimulated T cells were fractionated, and the ribosome-free and monosomal pools of mRNAs were resolved by gel electrophoresis. The 28S, 18S, and 5S rRNA components of the ribosome were visualized by ethidium bromide staining (Fig. 8a). Fractions 1–10 define the ribosome-free mRNA pool, whereas the ribosome-bound mRNAs are enriched in fractions 11–20 (39). The distribution of Bcl-xL mRNA transcripts associated with each pool was detected by Northern blot analysis using a Bcl-xL-specific cDNA probe. We observed that the Bcl-xL mRNAs derived from CD28−/− T cells were distributed equally between the free and polysome-bound pools (Fig. 8b), whereas CD28 costimulation of WT C57BL/6 T cells resulted in a significant redistribution of Bcl-xL mRNA transcripts from mRNPs into the translationally active polysome pool (Fig. 8c). This effect was dependent upon the capacity of CD28 to recruit PI3K, because no redistribution of Bcl-xL mRNA transcripts into the polysome pool was detected in the Y170F T cells (Fig. 8d). These data confirm that CD28 and its PI3K docking site Y170 are essential in augmenting the translational efficiency of Bcl-xL mRNA transcripts during T cell activation.

**CD28 induces redistribution Bcl-xL mRNAs from the pretranslational complex to translationally active polyribosomes**

Cytosolic mRNAs are held in two translationally distinct pools. mRNA species sequestered in the messenger ribonucleoprotein (mRNPs) pretranslational complex are inefficiently translated, whereas the translationally active mRNA fraction is associated with the polysome complex composed of the 40S (containing 18S and 5S rRNAs) and the 60S (containing 28S rRNA) ribosomal subunits (39). The mRNP particles and polyribosomes can be readily separated by sucrose gradient centrifugation, allowing these two pools of mRNAs to be effectively distinguished (40). We tested whether CD28 costimulation enhanced the pool of actively translated Bcl-xL transcripts in primary T cells. Polyribosome-bound Bcl-xL mRNAs was measured by Northern blot analysis after T cell activation with CD3 and CD28 from C57BL/6, CD28−/− or Y170F T cells. Cytoplasmic extracts from stimulated T cells were fractionated, and the ribosome-free and -bound pools of mRNA were resolved by gel electrophoresis. The 28S, 18S, and 5S rRNA components of the ribosome were visualized by ethidium bromide staining (Fig. 8a). Fractions 1–10 define the ribosome-free mRNA pool, whereas the ribosome-bound mRNAs are enriched in fractions 11–20 (39). The distribution of Bcl-xL mRNA transcripts associated with each pool was detected by Northern blot analysis using a Bcl-xL-specific cDNA probe. We observed that the Bcl-xL mRNAs derived from CD28−/− T cells were distributed equally between the free and polysome-bound pools (Fig. 8b), whereas CD28 costimulation of WT C57BL/6 T cells resulted in a significant redistribution of Bcl-xL mRNA transcripts from mRNPs into the translationally active polysome pool (Fig. 8c). This effect was dependent upon the capacity of CD28 to recruit PI3K, because no redistribution of Bcl-xL mRNA transcripts into the polysome pool was detected in the Y170F T cells (Fig. 8d). These data confirm that CD28 and its PI3K docking site Y170 are essential in augmenting the translational efficiency of Bcl-xL mRNA transcripts during T cell activation.

**Discussion**

CD28 has been shown to regulate gene expression either by stabilizing mRNA (41) or by regulating the activity of the NF-κB transcription factor (42, 43). Recent array analysis suggests that additional 6 h. c, Parental Jurkat T cells or Jurkat T cells containing stable transfected PTEN WT or PTEN G129R were electroporated in triplicate with pGL3–5′UTR Bcl-xL-luciferase vector for 18 h. Cells were grown for an additional 24 h with doxycyclin at 1 μg/ml for maximum PTEN induction. Cells were then stimulated with anti-CD3 (OKT3; 10 μg/ml) and/or anti-CD28 (9.3; 10 μg/ml) Abs for an additional 6 h. d, PTEN induction. Jurkat T cells or Jurkat T cells containing stable transfected PTEN WT or PTEN G129R were grown for 0, 6, 12, or 24 h with 1.0 μg/ml doxycyclin or for 24 h with 0, 0.2, 0.5, or 1.0 μg/ml doxycyclin for PTEN induction. The membranes were reprobed with anti-actin Ab. e, Parental Jurkat T cells, or Jurkat T cells containing stable transfected PTEN WT or PTEN G129R were electroporated in triplicate with pGL3-luciferase vector. Maximum PTEN expression was induced by doxycyclin. Cells were then stimulated with anti-CD3/28 Abs. Luciferase activity was measured in cell lysates, then normalized to β-galactosidase activity. β-Galactosidase plasmid was cotransfected to measure transfection efficiency. These experiments are representative of three independent experiments. f, Parental Jurkat T cells, or Jurkat T cells containing stable transfected PTEN WT tet-on were transfected with pGL3-luciferase or pGL3–5′UTR Bcl-xL-luciferase vectors. Maximum PTEN expression was induced by doxycyclin. Total RNA was extracted, and cDNAs were used in PCR with primers specific for luciferase and GAPDH to give a respective representation of the steady state level of mRNA expression.

CD3 and CD28 Abs showed a 2-fold increase in luciferase activity compared with cells that were unstimulated or stimulated with CD3 alone (Fig. 7c). EGFP+ primary T cells transfected with the luciferase control construct pGL3-Lac, did not exhibit differential luciferase activity after costimulation. These data show that CD28 costimulation removes the basal inhibited translational state of the 5′UTR of Bcl-xL, not only in Jurkat T cells, but also in primary murine T cells.
FIGURE 7. CD28 is essential in relieving the translational inhibitory effects associated with the 5'UTR of Bcl-xL. Purified T cells from C57BL/6 mice were stimulated with plate-bound anti-CD3/28 Abs (10 μg/ml). T cells were electroporated with the pEGFP and pGL3-luciferase constructs, then cultured for 2 h in medium before restimulated with plate-bound anti-CD3/28 Abs (10 μg/ml) for an additional 5 h. a, EGFP+ T cells were sorted. b, The percentage of EGFP+ T cells sorted out of total viable primary T cells was presented under different stimulating conditions and for the various vectors used. c, EGFP+ T cells (5 × 10^5) were lysed, and luciferase activity was measured. These data are representative of three independent experiments.
CD28 does not induce transcriptional targets distinct from CD3, but, rather, increases the amplitude of the CD3 transcriptional response, possibly through the regulation of NFAT (44). In this study we present evidence for a third mode of gene regulation by CD28 through the control of translational initiation.

CD28 induces the rapid expression of the prosurvival protein Bcl-xL (5–7). We present evidence that induction of Bcl-xL in primary T lymphocytes by CD28 requires the activation of PI3K (Fig. 9). Cells lacking CD28 or harboring a mutant form of CD28, which is uncoupled from PI3K, do not effectively up-regulate Bcl-xL, and have heightened sensitivity to early AICD. CD28-dependent activation of PI3K also protects T cells from Fas-mediated or gamma irradiation-induced apoptosis (12, 37). CD28-induced expression of Bcl-xL protein in peripheral T cells is exquisitely sensitive to PI3K inhibition. The activation of PI3K and the up-regulation of Bcl-xL after CD28 ligation are highly correlated, and both appear to be necessary to provide protection against each of these triggers of cell death. The observation that CD28 specifically regulates Bcl-xL, but not Bcl-2, suggests that Bcl-xL may act as a crucial survival switch in activated T cells after costimulation.

In this study we examined the role of CD28-mediated PI3K activation in protecting cells from early AICD. Early AICD is a perforin-dependent form of apoptosis that occurs before and independently of Fas-dependent death (45, 46). Although Fas-mediated AICD cultures require a minimum of 4 days to allow down-regulation of cFLIP before restimulation, perforin-dependent AICD...
FIGURE 9. CD28 regulates Bcl-xL expression via PI3K/mTOR-mediated translational initiation. CD28 activates and recruits PI3K upon T cell activation. PI3K recruits PKB by generating phosphatidylinositols. Activated PKB inhibits the GTPase activity of the Tsc1/2 complex, which leads to the accumulation of active Rheb-GTP. Rheb in its GTP-bound state activates the serine kinase mTOR, which regulates translational initiation through phosphorylation of 4E-BP1. Phosphorylation of 4E-BP1 releases eIF4E, which binds to the 5′ cap region of mRNA and recruits other factors of the translational machinery, including eIF4G and eIF4A, to initiate translation. This process increases the rate of translation of cap-dependent mRNAs, including Bcl-xL. This pathway can be blocked by LY294002, a PI3K inhibitor, and rapamycin, an mTOR inhibitor.

requires only 2 days of activation in culture and are thus independent of regulation by cFLIP (32, 45).

Although this study focused on the role of CD28 regulation of early AICD, the induction of PI3K activity by CD28 may prevent late AICD by mechanisms other than Bcl-xL up-regulation. Kirchhoff et al. (47) have shown that CD28 costimulation in primary T cells is associated with up-regulation of cFLIP, inhibition of Fas ligand mRNA, and protection of cells from Fas-mediated AICD. Consistent with this observation, PI3K activation may be required for the up-regulation of cFLIP (48, 49). In contrast to Kirchhoff et al. (47), Jones et al. (37) have demonstrated that cFLIP protein levels do not change in transgenic T cells overexpressing the constitutively active form of PKB upon CD3/CD28 costimulation. Thus, CD28 may regulate T cell longevity through a number of complementary mechanisms, including the coordinated expression of cFLIP, Fas ligand, and Bcl-xL.

We have shown that PI3K is required for Bcl-xL protein induction, although the specific p85/p110 isoforms that mediate this function remain to be identified. For example, T cells that are unable to activate p110β (p110β<sup>D910A</sup>) (50) are still capable of up-regulating Bcl-x<sub>L</sub> protein upon T cell costimulation (K. Okkenhaug, unpublished observation), suggesting that other PI3K catalytic subunits, such as p110α and p110β, may be involved in CD28-mediated Bcl-xL up-regulation. Although CD3-mediated PKB activation is abrogated in p110β<sup>D910A</sup> T cells, it is not yet known whether CD28 costimulation can overcome this block.

In this study we have shown that the expression of Bcl-xL mRNA is effectively induced by CD3 ligation alone and is not further enhanced by CD28 costimulation. Neither Ly294002 nor rapamycin was found to perturb Bcl-xL steady state mRNA levels at the doses needed to inhibit protein expression, indicating that the control of Bcl-x<sub>L</sub> protein expression by CD28 costimulation occurs at the post-transcriptional level. The expression of Bcl-x<sub>L</sub> after CD28-mediated costimulation was also sensitive to rapamycin, suggesting that Bcl-xL may be regulated at the translational level.

Efficient translation of cap-dependent mRNA species is regulated by growth factor signals, specifically through the PI3K/PKB pathway (22). Activation of PI3K leads to the production of PIP3 lipids anchored in the plasma membrane, which serve as ligands for PH-containing proteins such as phosphoinositide protein kinase-1 and PKB. The recruitment of these kinases to the plasma membrane initiates a signaling cascade that phosphorylates a number of downstream targets, including the Tsc1/Tsc2 complex. Tsc1 and Tsc2 form a physical complex, which function as a GTPase-activating protein (GAP) against the small GTPase Rheb. In its GTP-bound state, Ras homologue enriched in brain (Rheb) activates the serine/threonine protein kinase mTOR. Phosphorylation of Tsc2 by PKB inactivates the GAP activity of Tsc2, which results in the accumulation of Rheb-GTP and activation of mTOR (51, 52). The mTOR regulates cap-dependent mRNA translation in part, by phosphorylating 4E-BP1, an inhibitor of the cap-binding protein eIF4E, which recruits other initiation factors, such as the helicase, eIF4A, to the cap region (Fig. 9). Translation of mRNA species containing cap sequences in their 5′UTR regions is then initiated once the initiation complex eIF4F binds and unwinds the higher order hairpin structures present in the cap region (21).

We observed that the phosphorylation of 4E-BP1 was dependent on costimulation through CD28. In the absence of CD28, ligation of CD3 was insufficient to induce Bcl-xL protein expression or phosphorylate 4E-BP1, whereas CD28 costimulation of normal T lymphocytes potently increased both the Bcl-xL protein level and the level of 4E-BP1 phosphorylation. The induction of 4E-BP1 phosphorylation after CD28 costimulation was abrogated by inhibitors of either PI3K or mTOR, strongly suggesting that the downstream target of CD28 activation is the repressor of cap-dependent translation, 4E-BP1. We observed that transgenic expression of a constitutively active form of PKB (Gag-PKB) was associated with increased 4E-BP1 phosphorylation and increased Bcl-xL expression (29). We propose that CD28, through the activation of the PI3K/mTOR pathway, induces phosphorylation of 4E-BP1, which, in turn, increases the efficiency of translational initiation of the Bcl-xL transcript.

To directly test the capacity of CD28 costimulation to regulate translation of Bcl-xL, we measured the effect of CD28 ligation on
the translational efficiency of the Bcl-xL 5′UTR using a luciferase reporter gene system in Jurkat and primary T cells. Whereas anti-CD3 Ab had little effect on increasing the translation of the Bcl-xL 5′UTR reporter, CD28 costimulation substantially increased the luciferase activity of the reporter gene in both Jurkat and primary T cells. This effect was suppressible by 4E-BP1, but not by BP1-4E, demonstrating that the translation induction of Bcl-xL by CD28 is dependent upon the cap-binding protein eIF-4E. The induction of Bcl-xL translation by CD28 was completely repressed by the forced expression of PTEN, but not by a catalytically inactive form of PTEN. This demonstrates that the capacity of CD28 to stimulate the production of Pi3 phospholipids necessary in the regulation of Bcl-xL translation by CD28. Therefore, we have demonstrated, both genetically and pharmacologically, that PI3K is the proximal control point required for CD28-mediated translational regulation of Bcl-xL.

It has been estimated that ~13% of the mRNA species in activated T cells are translationally activated or repressed (39). To determine whether CD28 induced the pool of actively translated Bcl-xL mRNA transcripts, we used sucrose gradient centrifugation to measure the proximal control point required for CD28-mediated translation of Bcl-xL.

Recent studies have shown that the mTOR pathway may also be modulated by nutrients, such as amino acids, ATP, and phosphatic acid (21, 53–55). The mTOR thus serves as a checkpoint for the physical redistribution of Bcl-xL mRNA transcripts onto the translationally active polysomes required for the activation of PI3K by CD28.

We have shown that Bcl-xL protein expression is dependent on signals emanating from both the TCR and CD28. Whereas the TCR principally determines transcription of the Bcl-xL locus, CD28 is critical in relieving the inhibitory machinery governing the translation of Bcl-xL mRNA. Our data may explain in part the molecular basis of the immunosuppressive effect of rapamycin in attenuating Bcl-xL expression and, hence, the longevity of the T cell response.

Acknowledgments
We thank Drs. Michael P. Bova, Chuangwu Wu, Richard Miller, Philippe Foussier, and Nahum Sonenberg for advice and review of the manuscript.

References


