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A CD2/CD28 chimeric receptor triggers the CD28 signaling pathway in CTLL.2 cells

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We are evaluating strategies to enhance the in vivo proliferation and function of adoptively transferred antigen-specific T cells. Although the CD28 costimulatory pathway is important for T cell activation and proliferation, the expression of the ligands for CD28 is highly restricted. We have generated a chimeric receptor composed of the signaling domains of CD28 and the extracellular domain of CD2 which binds the widely expressed ligand CD58. The CD2/CD28 chimeric receptor was introduced into CTLL.2 cells via retrovirus infection and was shown to be expressed on the cell surface. By monitoring early and late components of the CD28 signaling pathway, the chimeric receptor was demonstrated to trigger the CD28 pathway in response to CD2 cross-linking. The possible utility of the CD2/CD28 chimeric receptor for adoptive immunotherapy is discussed.

Keywords: *CD2; CD28; costimulation; adoptive immunotherapy*

Introduction

The adoptive transfer of *in vitro* expanded lymphokine activated killer cells (LAK), tumor infiltrating lymphocytes (TILs) and cytotoxic T lymphocytes (CTL) has been demonstrated to mediate tumor regression or resistance to certain infectious diseases.[1,2] However, optimal T cell proliferation and cytotoxic function of adoptively transferred T cells requires systemic administration of IL-2 which is associated with severe toxicity.[3] In addition, large numbers of adoptively transferred T cells are required for therapeutic efficacy which may severely impact the commercialization of such therapies. We are developing strategies to enhance the *in vivo* proliferation and function of antigen-specific CTL clones. Such modified T cells will reduce the number of adoptively transferred CTLs required for clinical significance and eliminate the need for large systemic doses of IL-2.

The activation and proliferation of T cells requires multiple signals. Engagement of the T cell receptor (TCR) alone is insufficient to fully activate T cells and can result in induction of anergy. A second signal or costimulation is required to induce optimal T cell proliferation and cytokine secretion.[4–6] CD28 has been demonstrated to fulfill the costimulatory requirement and play an important role in the activation and proliferation of T cells. Stimulation of T cells through the TCR and CD28 results in T cell proliferation and increased expression of a wide variety of genes including IL-2.[7–10] CD28 signaling has also been shown to induce cell survival genes including Bcl-X_L and thus may play an important role in protecting T cells from apoptosis.[11] The induction of cell survival genes suggests that in addition to the important role CD28 plays in the activation of T cells, it may be critical to sustain clonal expansion and establish immunologic memory.

A strength of signal theory has been proposed, suggesting a role for CD28 in the activation of T cells.[10] This model of costimulation proposes that under conditions of low antigen density, CD28 signaling is critical for T cell activation, expansion and cytokine production. When antigen density is high, however, CD28 signaling may be dispensable. Consistent with this hypothesis, recent reports demonstrate that CD28 costimulation significantly reduces the number of occupied TCRs required to induce T cell activation.[12,13]

In the past several years a variety of tumor antigens have been described[14] suggesting that spontaneous tumors which appear to be non-immunogenic can serve as targets for T cell responses. Numerous factors including lack of costimulation have been implicated to contribute to the escape of tumors from immune surveillance.[15,16] Lack of costimulation may also impair the function of adoptively transferred T cells by increasing the antigen density requirements for T cell activation and limiting clonal expansion. Two ligands for CD28 have been described, B7–1[17] and B7–2,[18,19] which are members of the immunoglobulin gene superfamily. The expression of these ligands is highly restricted to professional antigen presenting cells[10] and absent from the majority of tumors. These observations led to the development of costimulation-based tumor vaccines. Numerous studies have demonstrated that the introduction of B7–1 and/or B7–2 into a variety of murine tumors results in rejection of the modified tumor and in some cases protects against future challenge with unmodified tumor.[9,20,21] Tumor clearance and protection against future challenge has been demonstrated to be mediated by $CD8^+$ T cells. While this approach has provided encouraging results, it is not clear if pre-existing tumors can be eradicated by this method.

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An alternative strategy is to modify antigen-specific T cells such that the CD28 costimulatory pathway is triggered within a B7-negative environment. To that end, we have generated a chimeric receptor composed of the extracellular domain of CD2 and the transmembrane and cytoplasmic domains of CD28. CD2 is a glycoprotein expressed on T cells and has been implicated to improve adhesion between T cells and target cells as well as deliver an activation signal.[22] The ligand for CD2 is CD58 which, unlike B7–1 or B7–2, is expressed on all cell types in humans.[23] We present preliminary data on the CD2/CD28 chimeric receptor which demonstrate that in CTLL.2 cells the CD2/CD28 chimeric receptor can trigger the CD28 signaling pathway and induce responses associated with T cell activation and proliferation.

Results

Generation of the CD2/CD28 chimeric receptor

The extracellular domain of hCD2 and the transmembrane and cytoplasmic domains of hCD28 were amplified by PCR and cloned into a retroviral vector under the control of the MMLV LTR. The vector also contains an internal ribosome entry site (IRES) followed by the *HYTK* gene which permits selection in the presence of hygromycin B (see Materials and methods and Figure 1). A high titer PA317 viral producer line was generated which contained a single intact provirus and expressed CD2/CD28 (data not shown).

Retroviral infection of CTLL.2 and expression of CD2/CD28

CTLL.2 cells are a murine IL-2-dependent T cell line.[24] CTLL.2 cells were infected with the CD2/CD28 containing retrovirus by cocultivation with irradiated viral producer cells and selected in the presence of 300 U/ml hygromycin B. Drug-resistant clones were screened initially by PCR for the hygromycin phosphotransferase gene. Subsequently, Southern blots demonstrated the clones contained a single intact provirus (data not shown).

Expression of the CD2/CD28 chimeric receptor by the drug-resistant clones was determined by flow cytometry and Western blot analyses for hCD2. Greater than 50% of the drug-resistant CTLL.2 clones expressed CD2/CD28 at levels detectable by both methods. One clone (clone 27) which expressed high levels of CD2/CD28 and mCD3 levels similar to the parental (Figure 2) was expanded and used in all analyses. The CTLL.2 parental cells and clone 27 do not express mCD28 at levels detectable by flow cytometry (Figure 2). The Western blot reveals that CD2/CD28 migrates as heterogeneous-sized bands ranging in size from 35 to 50 kDa (Figure 2). Endogenous hCD2 also displays size heterogeneity ranging from 50 to 65 kDa. This heterogeneity in size is apparently due to differing levels of glycosylation (Ref. 25 and data not shown). We have observed that only the slower migrating CD2/CD28 species is expressed on the cell surface (data not shown).

Association of p85 subunit of PI3-kinase with CD2/CD28

To determine if the CD2/CD28 chimeric receptor was capable of triggering the CD28 signal, two assays were performed to examine events associated with CD28 signaling. Phosphatidylinositol 3-kinase (PI3-kinase) is a ubiquitously expressed heterodimeric lipid kinase which is activated by most protein tyrosine kinase-dependent

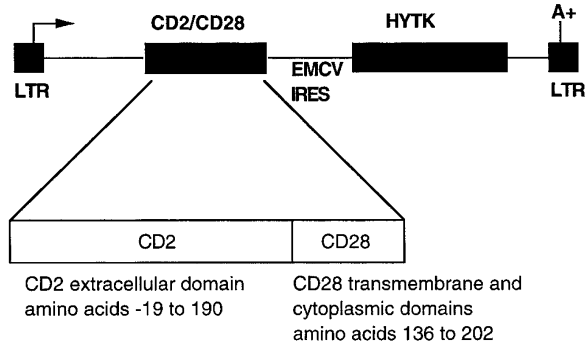

Figure 1 *Schematic drawing of the retroviral vector containing the CD2/CD28 chimeric receptor. CD2/CD28 and HYTK are transcriptionally regulated by the retroviral LTR. The arrow denotes initiation of transcription. The retroviral polyadenylation signal (A+) was utilized. The regions from CD2 and CD28 which were fused to generate the chimeric receptor are indicated.*

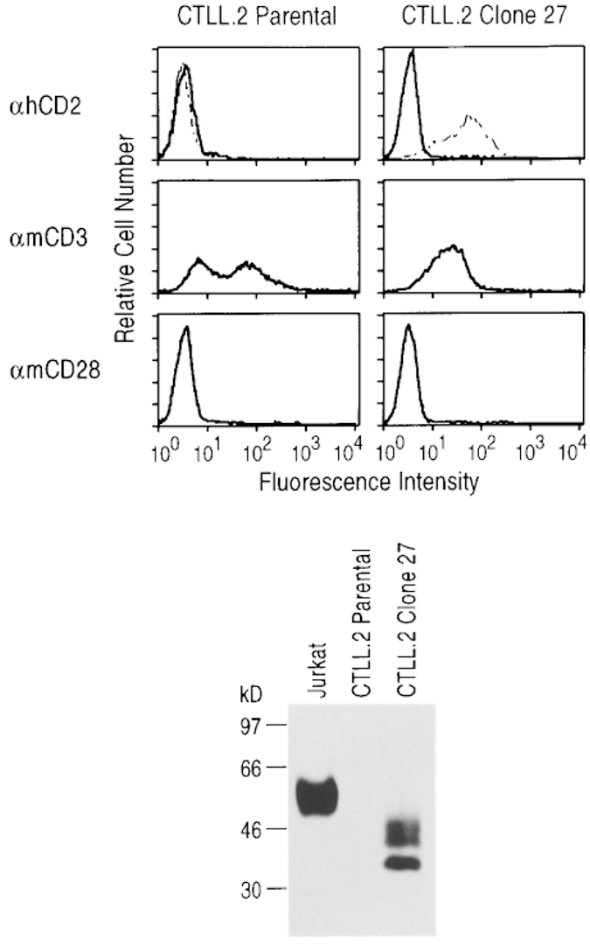

Figure 2 *Expression of CD2/CD28 in CTLL.2 cells. CTLL.2 parental and clone 27 cells were analyzed by flow cytometry for expression of hCD2 (CD2/CD28), mCD3 and mCD28 as described in Materials and methods. CD2/CD28 expression was also detected in clone 27 cells by a CD2 Western blot. Jurkat cells which express high levels of hCD2 were used as a positive control.*

receptors.[26] The p85 subunit of PI3-kinase contains two scr homology domains which can associate with a tyrosine phosphorylated YMXM domain. Several groups have demonstrated that CD28 cross-linking results in the rapid association of the p85 subunit with the YMNM motif within the cytoplasmic domain of CD28[27,28] and is believed to be one of the earliest signaling events in the CD28 signaling pathway.

We examined if p85 could associate with the CD2/CD28 chimeric receptor following cross-linking with anti-hCD2. CTLL.2 cells are negative for mCD28 so EL-4 cells were used as a positive control for the immunoprecipitation and Western blot. Immunoprecipitation of CD28 from EL-4 cells co-immunoprecipitated low levels of the p85 subunit of PI3-kinase (Figure 3). Cross-linking of EL-4 cells with anti-mCD28 before immunoprecipitation significantly increased co-immunoprecipitation of p85 (Figure 3) which is consistent with previously published results.[27,28] The mAb 35.1 recognizes an epitope on the extracellular domain of hCD2 and thus recognizes CD2/CD28. Immunoprecipitation with mAb 35.1 resulted in co-immunoprecipitation of p85 at background levels from unstimulated and mAb 35.1 cross-linked EL-4 cells (Figure 3). Similarly with CTLL.2 parental and clone 27 cells, immunoprecipitation of hCD2 resulted in minimal co-immunoprecipitation of p85. Cross-linking with mAb 35.1 before immunoprecipitation of hCD2 resulted in high levels of p85 co-immunoprecipitation with clone 27 cells but not parental CTLL.2 cells (Figure 3). Thus, under the appropriate stimulation conditions, p85 co-immunoprecipitated with CD2/CD28 as with endogenous CD28. This result demonstrates that the first known step of the CD28 signaling pathway is initiated by CD2/CD28 in response to anti-hCD2 cross-linking.

Induction of Bcl-X_L expression in CD2/CD28-infected CTLL.2 cells

The association of p85 with CD2/CD28 represents an early event in the CD28 signaling pathway. The components of the CD28 signaling pathway are not well characterized but CD28 signaling modulates expression of a large number of genes through stabilization of mRNA and transcriptional up-regulation.[10,29] One such gene is Bcl-X_L which plays an important role in protecting T cells from apoptosis. Bcl-X_L is not expressed in resting T cells but can be induced in T cells by sub-optimal anti-CD3 stimulation plus anti-CD28 stimulation.[11] We tested whether signaling through CD2/CD28 and the TCR could induce Bcl-X_L expression. CTLL.2 parental and clone 27 cells were stimulated with 500 ng/ml anti-mCD3, 10 μg/ml anti-hCD2 or a combination of the two. Cells were incubated 20 h following stimulation and Bcl-X_L expression assessed by Western blot. Bcl-X_L expression was not detected in either unstimulated parental or clone 27 cells (Figure 4). Parental and clone 27 cells stimulated with anti-hCD2 alone did not induce Bcl-X_L while stimulation with anti-mCD3 alone induced low levels of Bcl-X_L (Figure 4). The combination of anti-mCD3 and anti-hCD2 stimulation induced Bcl-X_L in parental cells to the level seen with anti-mCD3 alone. However, in clone 27 cells the combination induced high levels of Bcl-X_L expression. Thus, TCR and CD2/CD28 cross-linking modulated expression of Bcl-X_L as previously demonstrated for TCR and CD28 cross-linking. This result demonstrates that CD2/CD28 is capable of providing CD28 costimulation in conjunction with signaling through the TCR.

Discussion

A variety of ligand–receptor pairs have been described which are involved in T cell activation. LFA-1 and CD2 are believed to increase avidity between T cells and APCs and are capable of signaling in response to the appropriate ligands.[30,31] However, the signaling pathways initiated by these receptors are redundant to that initiated by the TCR and thus, their role in T cell activation is to augment the TCR signal.[32] CD28, however, has been demonstrated to play an important role in T cell activation and proliferation. The observation that the CD28 signaling pathway resulted in cyclosporin A and FK506-resistant IL-2 gene expression demonstrated the CD28 signal to be biochemically distinct from the signaling pathway initiated by the TCR.[33]

Due to the highly restricted expression of the ligands for CD28, we have generated a chimeric receptor capable of triggering this important costimulatory signal in

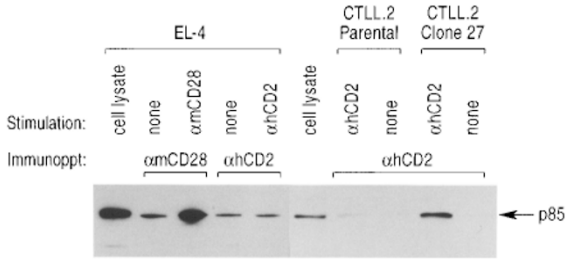

Figure 3 *Association of the p85 subunit of PI3-kinase with CD2/CD28. Cells were cross-linked and immunoprecipitations performed with the indicated antibodies as described in Materials and methods. EL-4 cells served as a positive control for p85 association with CD28 following CD28 cross-linking. Cell lysates served as a positive control for the p85 Western blot.*

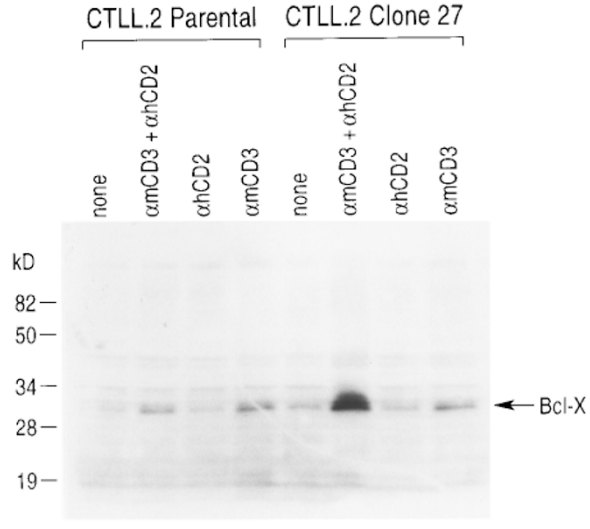

Figure 4 *Induction of Bcl-X_L expression following cross-linking of mCD3 and CD2/CD28. Cells were cross-linked with the indicated antibodies and 20 h after treatment the cells were lysed and Bcl-X_L expression analyzed by Western blot.*

environments lacking B7 expression. The chimeric receptor contains the ligand binding domain from CD2 and the transmembrane and signaling domains from CD28. We have demonstrated that the chimeric receptor triggers the CD28 signaling pathway in response to hCD2 cross-linking.

Currently, we are modifying antigen-specific $CD8^+$ T cell clones directed against tumor or viral antigens for use in adoptive immunotherapy with the CD2/CD28 chimeric receptor. A number of issues need to be addressed to determine if the CD2/CD28 chimeric receptor will be useful for adoptive immunotherapy. One important issue concerns the response of previously activated T cell clones to CD28 costimulation. In our hands antigen-specific $CD8^+$ clones do respond to stimulation through the TCR and CD28 as determined by proliferation assays and IL-2 secretion (data not shown). Thus, we anticipate that CD2/CD28 will have a functional impact on the modified T cell clones. In response to CD58 and the appropriate antigen, modified T cells will initiate three signaling pathways. Antigen will provide TCR signaling while CD58 will trigger both the CD2 pathway via endogenous CD2 and the CD28 pathway through the CD2/CD28 chimeric receptor. The signaling pathways of CD2 and CD28 have been shown to be synergistic with respect to T cell activation and proliferation *in vitro*[34,35] and Li *et al*[36] have demonstrated similar results *in vivo* in a tumor rejection model. Introduction of the ligands for CD2 and CD28 individually or together in two poorly immunogenic murine tumors resulted in a significant immune response only when both ligands were present.[36]

Our approach to provide costimulation is to modify antigen-specific CTL rather than the target cells. By this strategy, large numbers of modified CTLs capable of enhanced reactivity to all target cells can be delivered via adoptive immunotherapy. This should provide a significant advantage over approaches which modify only a small percentage of target cells by the addition of B7–1 or B7–2.

To achieve activation and proliferation of CD2/CD28 modified T cells, the target cells must express the appropriate antigen in the context of MHC class I and CD58. CD58 is expressed on all cell types and we have examined several primary tumors and tumor-derived cells lines and have found all to express CD58 and MHC class I (Table 1). Therefore, due to the signals delivered by CD2/CD28 and endogenous CD2, modified CTLs should be activated via a larger variety of antigen expressing cells (ie low antigen density). In addition to decreasing requirements for activation, the induction of Bcl-X_L by CD2/CD28 stimulation may act to increase CTL longevity by protecting cells from apoptosis. It is anticipated that these phenotypic changes will reduce the number of adoptively transferred CTLs required for clinical efficacy and eliminate the need for large systemic doses of IL-2.

Materials and methods

Cells

CTLL.2 cells[24] were cultured in IMDM supplemented with 10% heat inactivated fetal calf serum, 15 U/ml IL-2 (Chiron, Emeryville, CA, USA), 1 × MEM non-essential amino acids, 1 mM sodium pyruvate, 5 μM 2-mercaptoethanol, 10 mM Hepes, 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. Ψ2[37] and PA317 cells[38] were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin.

Generation of CD2/CD28 chimeric receptor

The extracellular domain of hCD2 (cDNA a gift from Brian Seed, Massachusetts General Hospital) and the transmembrane and cytoplasmic domains of hCD28 (cDNA a gift from Craig Thompson, University of Chicago) were amplified by PCR. Primers were utilized to amplify amino acids –19 to 190 of CD2[39] and amino acids 136 to 202 of CD28.[40] The amplified fragments were ligated in a SK vector (Stratagene, La Jolla, CA, USA). The CD2/CD28 chimeric receptor was subsequently subcloned into a retroviral vector under the transcriptional control of the MMLV LTR. Immediately downstream of the CD2/CD28 chimeric receptor is an EMCV internal ribosome entry site (IRES) followed by a *HYTK* gene which permits positive selection on hygromycin B and negative selection on ganciclovir.[41]

Generation of CD2/CD28 and retroviral producer line

Retroviral producer lines were generated by transient transfection of Ψ2 cells followed by transinfection of PA317 cells as previously described.[41] Clones were expanded in the presence of 300 U/ml hygromycin B (Calbiochem, La Jolla, CA, USA). Viral producer lines were characterized for titer on NIH 3T3 cells and Southern blot analyses were performed to determine copy number and viral integrity. A high titer viral producer line containing a single intact provirus was used for infection of CTLL.2 cells.

Retroviral infection of CTLL.2 cells

Six-well cluster dishes were seeded with 2.5×10^5 PA317 CD2/CD28 viral producer cells in 2.5 ml of complete medium. Cells were grown to 75% confluency and irradiated at 30 Gy. Actively proliferating CTLL.2 cells were added to the producer cells in 2.5 ml of complete medium supplemented with 30 μg/ml DEAE-dextran (Sigma, St Louis, MO, USA). The CTLL.2 cells were cultivated in the presence of the irradiated viral producer cells for 20 h. CTLL.2 cells were harvested and replated to eliminate contaminating viral producing cells followed by cloning at 10 000 cells per well in 96-well flat-bottom plates containing 200 μl IMDM complete medium supplemented with 300 U/ml hygromycin B.

Flow cytometry

mCD3, mCD28 and hCD2/CD28 expression was monitored by immunofluorescence staining. 1×10^6 Cells in 100 μl buffer were incubated with anti-mCD3-FITC (Pharmingen, San Diego, CA, USA), anti-mCD28 (Pharmingen) or 20 μl of anti-hCD2 hybridoma supernatant (ATCC HB 195; Rockville, MD, USA) for 20 min at 4°C. Cells were washed twice. The cells stained with anti-mCD28 or anti-hCD2 were incubated with goat anti-hamster IgG-FITC or goat anti-mouse IgG-FITC (Cappel, Durham, NC, USA) respectively. After incubation, cells were washed and analyzed on a Becton Dickinson FACscan (San Jose, CA, USA).

CD2 Western blot

Whole cell lysates were resolved on 10% polyacrylamide gels and transferred to Immobilon P (Millipore, Bedford,

Table 1 CD58 expression on tumor cells and cell lines

Patient	*Tissue type*	*Class I expression*	*CD58 expression*
001 AEW	Colon	Positive	Positive
A03 FMC	Colon	Positive	Positive
B03 JP	Colon	Positive	Positive
A05 RCT	Colon	Positive	Positive
C01 MC	Colon	Positive	Positive
Cell line	*Tumor type*	*Class I expression*	*CD58 expression*
LoVo	Colon Adenocarcinoma	Negative (γIFN induced)	Positive
U-937	Lymphoma	Positive	Positive
T84	Colon Adenocarcinoma	Positive	Positive
HT-29	Colon Adenocarcinoma	Positive	Positive
SK-Co-1	Colon Adenocarcinoma	Positive	Positive
SKBr-3	Breast Adenocarcinoma	Positive	Positive
LT174	Colon Adenocarcinoma	Positive	Positive
BT 549	Breast Ductal carcinoma	Positive	Positive

MA, USA). The membrane was incubated with media supernatant from HB195 (ATCC) diluted 1:5 containing anti-hCD2 followed by goat anti-mouse IgG diluted 1:5000 (Cappel). CD2 was visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham, Arlington Heights, IL, USA).

PI3-kinase assay

EL-4, CTLL.2 parental and clone 27 cells (2×10^7 cells in 0.5 ml serum-free IMDM) were incubated in the presence of 10 μg/ml anti-hCD2 or anti-mCD28 for 3 min at 37°C followed by 10 μg/ml goat anti-mouse IgG (Cappel) or goat anti-hamster IgG (Cappel) for 20 min at 37°C. Cells were pelleted, washed once with cold PBS, resuspended in 0.5 ml NP-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 1% NP40) and incubated on ice for 30 min. Lysates were centrifuged at 14 000 *g* for 5 min and post-nuclear supernatants incubated with 10 μg/ml anti-hCD2 or anti-mCD28 and 10% w/v protein G-Sepharose beads (Sigma) at 4°C for 3–4 h. The immunoprecipitates were washed extensively with lysis buffer and then denatured in disruption buffer (12 mM Tris, pH 6.8, 2% SDS, 3% glycerol and 0.15% β-mercaptoethanol). Proteins were resolved on a 10% acrylamide gel and transferred to Immobilon P. Whole cell lysates (5×10^4 cells) were included as a positive control for the p85 Western blot. Anti-p85 immunoblotting was performed as previously described.[28]

Induction of Bcl-X

CTLL.2 parental and clone 27 cells (5×10^5 cells in 0.5 ml serum-free IMDM) were incubated in the presence of 500 ng/ml anti-mCD3, 10 μg/ml anti-hCD2, or both for 3 min at 37°C followed by 10 μg/ml goat anti-hamster IgG (for CD3) and/or goat anti-mouse IgG (for CD2) for 20 min at 37°C. Cells were then diluted to 3.0 ml with complete media and incubated for 20 h at 37°C. Cells were harvested, lysed in disruption buffer, run on a 4–20% acrylamide gradient gel, and transferred to Immobilon P. The membrane was incubated in the presence of anti-Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 followed by goat anti-mouse Ig (Cappel) diluted 1:5000. Bcl-X_L was visualized by ECL.

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