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The NF-κB Cascade Is Important in Bcl-x_L Expression and for the Anti-Apoptotic Effects of the CD28 Receptor in Primary Human CD4$^+$ Lymphocytes[1]

Ali Khoshnan,* Charles Tindell,* Isett Laux,* David Bae,* Brydon Bennett,† and Andre E. Nel[2]*

We explored the role of the NF-κB pathway in the survival of primary human CD4$^+$ T lymphocytes during CD28 costimulation. Transduction of proliferating CD4$^+$ T cells with a tetracycline-regulated retrovirus encoding for a dominant-interfering, degradation-resistant I-κBα (inhibitor of κBα factor) mutant induced apoptosis. Using DNA arrays, we show that Bcl-x_L features as a prominent anti-apoptotic member among a number of early CD28-inducible genes. A 1.2-kb segment of the proximal Bcl-x_L promoter, linked to a luciferase reporter, responded to CD3/CD28 stimulation in Jurkat cells. Mutation of an NF-κB site around −840 decreased, while ectopic expression of I-κB kinase-β (IKKβ) enhanced reporter gene activity. Na^+-salicylate and cyclopentenone PGs, direct inhibitors of IKKβ, interfered in the activation of the Bcl-x_L promoter and induced apoptosis in CD28-costimulated CD4$^+$ T cells. Moreover, salicylate blocked nuclear localization of NF-κB factors that bind to the NF-κB binding site in the Bcl-x_L promoter, as well as the expression of Bcl-x_L protein. HuT-78, a lymphoblastoid T cell line with constitutive NF-κB activity, contained elevated levels of Bcl-x_L protein and, similar to proliferating CD4$^+$ T cells, was resistant to apoptotic stimuli such as anti-Fas and TNF-α. In contrast, the same stimuli readily induced apoptosis in a Jurkat T cell clone with no detectable Bcl-x_L expression. Jurkat BMS2 cells also differed from HuT-78 in collapse of mitochondrial membrane potential and superoxide generation in the mitochondrium. Taken together, these data demonstrate that CD3/CD28-induced activation of IKKβ and expression of Bcl-x_L promote the survival of primary human CD4$^+$ T lymphocytes. ***The Journal of Immunology,* 2000, 165: 1743–1754.**

Homeostasis in the peripheral T cell compartment is dependent on a complex network of cell surface receptors, cytoplasmic signaling components, and transcription factors that regulate growth, differentiation, and apoptosis of these cells (1–3). We are particularly interested in the role of the NF-κB cascade on genes that regulate above biological responses in mature T cells. In this regard, we have recently shown in primary human CD4$^+$ lymphocytes that the TCR is critically dependent on the CD28 accessory receptor for the activation of the multisubunit inhibitor of κB (I-κB)[3] kinase (IKK) complex, which induces I-κBα and I-κBβ phosphorylation (4, 5). Phosphorylation of these inhibitory proteins leads to degradation and the release of Rel/NF-κB proteins from the cytosol to the nucleus (6, 7). IL-2 is among the genes that are induced by NF-κB transcription factors and contains key NF-κB regulatory sites in its promoter (4, 5, 8). While IL-2 plays a role in the effect of the CD28 receptor on T cell growth and prevention of anergy, CD28 also assists in the expression of genes that regulate cell viability (9–13). In this communication, we will focus on the role of the CD28 receptor and the NF-κB cascade in preventing activation-induced cell death in human T lymphocytes.

The Rel/NF-κB family of proteins has diverse and opposing effects on apoptosis regulation in different tissues (14–19). While, for instance, Rel/NF-κB factors suppress apoptosis in immature B lymphocytes and developing thymocytes, c-Rel is highly expressed in cells undergoing apoptosis in developing embryos (20, 21). However, analysis of c-Rel-deficient mice does not support an obligatory role for c-Rel in apoptosis (22). While there are no definitive studies looking at the role of NF-κB pathway in human peripheral blood T lymphocytes, murine studies indicate that the NF-κB pathway has anti-apoptotic effects in mature T lymphocytes (1, 23). Included among the anti-apoptotic genes that may be NF-κB regulated and that have demonstrated links to the CD28 receptor is Bcl-x_L (24, 25). Both the human and mouse Bcl-x_L promoters contain NF-κB binding sites that have been shown to be activated in B cell lines by CD40 or in a T cell line by Tax overexpression (26, 27).

We wished to determine which apoptosis-related genes are induced by CD28 costimulation in primary human CD4$^+$ lymphocytes, and whether activation of the NF-κB pathway plays a role in the expression of anti-apoptotic genes. We show through the use of cDNA expression arrays that while CD28 costimulation is associated with early expression of several pro-apoptotic genes, there is prominent expression of only one anti-apoptotic gene, namely Bcl-x_L. Interference in the NF-κB cascade in CD4$^+$ lymphocytes

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[3] Abbreviations used in this paper: I-κB, inhibitor of κB factor; cIAP, cellular inhibitor of apoptosis protein; 15dPGJ$_2$, 15-deoxy-Δ^{12-14}-PGJ$_2$; DiOC$_6$, dihexyloxacarbocyanine iodide; DN, dominant-negative; GFP, green fluorescent protein; HE, hydroethidine; IKK, I-κB kinase; P+I, PMA plus ionomycin; PI, propidium iodide; tet, tetracycline; tTA, tet-repressible transactivating; $\Delta\psi_m$, mitochondrial membrane potential.

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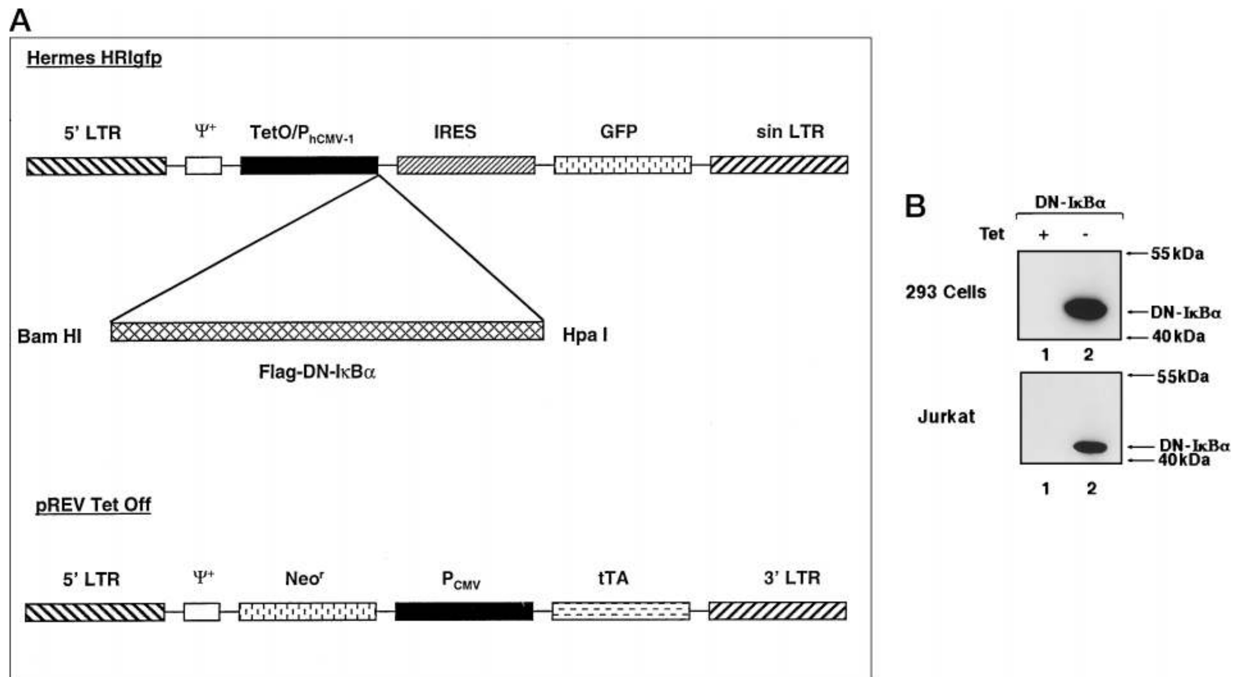

FIGURE 1. Tet-regulated expression of DN/I-κBα in 293-T and Jurkat T cells transduced with recombinant retroviruses. *A*, Physical map of the tTA and DN/I-κBα retroviral vectors. *B*, Western blot analysis of lysates from 293-T and Jurkat cells transduced with the tTA and DN/I-κBα retroviruses in the presence and absence of tet. The 293-T and Jurkat cells were sequentially infected with both retroviruses, as described in *Materials and Methods*. A total of 50 μg of each lysate was examined for DN/I-κBα expression by Western blot analysis using anti-Flag Ab (1:1000), followed by sheep anti-mouse (1:2000) conjugated to HRP. Reactive proteins were detected by enhanced chemiluminescence (ECL). The arrow points to the 44-kDa DN/I-κBα protein. Longer exposure did show a faint band of similar size in samples incubated with tet (not shown).

by a retrovirally expressed, degradation-resistant I-κBα mutant or inhibitory drugs induced apoptosis in parallel with decreased Bcl-x_L expression. Furthermore, NF-κB-mediated activation of Bcl-x_L in CD4$^+$ T cells is influenced by IKKβ function. CD3/CD28 stimulation as well as ectopic expression of IKKβ regulated Bcl-x_L promoter activity. In addition, Bcl-x_L expression correlated with resistance of proliferating CD4$^+$ T cells to a variety of apoptotic stimuli. This indicates that CD3/CD28-mediated activation of IKKβ and subsequent expression of Bcl-x_L may coordinate the life span of CD4$^+$ T cells.

Materials and Methods

Reagents

OKT3 (anti-CD3) was obtained from Ortho Pharmaceuticals (Raritan, NJ), and the 9.3 mAb (anti-CD28) was generously provided by Bristol-Meyer Squibb (Princetown, NJ). For the purpose of cellular activation, these primary stimulating Abs were cross-linked with mAb 187.1 (Bristol-Meyer Squibb). Anti-Bcl-x_L Abs and human TNF-α were purchased from PharMingen (San Diego, CA), while anti-cIAP2 goat Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag (M2) Abs were obtained from Sigma (St. Louis, MO). HRP conjugated to protein A was from Amersham (Arlington Heights, IL). Tosyl-activated magnetic beads and M-450 anti-CD4 beads were purchased from Dynal (Great Neck, NY). PMA (P), ionomycin (I), pyrrolidine-dithiocarbamate, and sodium salicylate (Na^+) were from Sigma. Anti-Fas mAb (CH11) was obtained from MBL (Nagoya, Japan). The annexin V/propidium iodide (PI) staining kit system was purchased from Trevigen (Gaithersburg, MD). Retronectin was from Takara Biomedicals (Otsu, Shiga, Japan), and 3,3′ dihexyloxacarbocyanine iodide ($DiOC_6$) and hydroethidine (HE) were obtained from Molecular Probes (Eugene, OR). Staurosporine and cycloheximide were purchased from ICN (Costa Mesa, CA). Prostaglandins PGA_1, PGB_2, and 15-deoxy-Δ^{12-14}-PGJ_2 (15dPGJ_2) were purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture and preparation of primary CD4$^+$ T cells

Jurkat (clone BMS2) and HuT-78 T cell lines were grown in RPMI medium, supplemented with 10 mM HEPES, 10% FCS, 2 mM glutamine, 100 U penicillin, and 100 μg streptomycin/ml. The 293-T epithelial cells were cultivated in DMEM containing 10% FCS, 2 mM glutamine, 100 U penicillin, and 100 μg streptomycin/ml. For preparation of CD4$^+$ T cells, mononuclear cells were isolated from human peripheral blood by density centrifugation and depletion of adherent cells on plastic culture dishes. CD4$^+$ T cells were positively selected with anti-CD4 Dynabeads according to manufacturer's instructions (Dynal). This yielded a T cell subset that was >98% positive for the CD4 marker, as determined by dual color CD4/CD8 flow cytometry. Isolated CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAb coupled to tosyl-activated magnetic beads, as previously described (5, 28). Cells were replenished with 40 U/ml rIL-2 (Chiron, Emeryville, CA) on day 3, and allowed to complete their growth cycle over the course of 12–13 days. At this point, IL-2 and beads were removed, and cells allowed to return to their resting state over a 48-h time period. Resting vs activated state was assessed by cell size analysis in a Coulter counter (Coulter Pharmaceutical, Palo Alto, CA). Cells were subjected to flow cytometry for a second time to confirm that >95% of the cells remained CD4$^+$.

Construction and preparation of a tetracycline (tet)-regulated DN/I-κBα-expressing retroviral vector

The mutant I-κBα construct, with mutational alteration of Ser^{32} and Ser^{36} to Ala residues, has been previously described (7). The Hermes HRIgfp tet-regulated retroviral vector was kindly provided by Dr. Helen Blau (Stanford University, Palo Alto, CA) (29). The backbone, in addition to long terminal repeats and packaging signal, contains six copies of a tet operon (Tet O) linked to a minimal CMV promoter (P_{hCMV-1}) (see Fig. 1*A*). Green fluorescent protein (GFP) is expressed from an internal ribosome entry site inserted downstream of the multiple cloning site (29). N-terminal Flag-tagged DN/I-κBα generously provided by Dr. G. Cheng (University of California at Los Angeles), was directionally cloned into the *Hpa*I-*Bam*HI site of the Hermes vector. In-frame insertion was confirmed by DNA sequencing (see Fig. 1*A*). The second retroviral vector (pREV Tet-off) encoding for the tet-repressible transactivating (tTA) protein was obtained from Clontech (Palo Alto, CA). Individual retroviruses were produced in the 293-Ampho (American Type Culture Collection, Manassas, VA) packaging cell line. Briefly, packaging cell lines were plated in 10-cm dishes at a confluency of ~70% and transfected with 20 μg of the Hermes or pREV Tet-off vector by calcium-phosphate precipitation procedures in the presence of 25 μM chloroquine. After overnight incubation at 37°C, the

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culture media were replaced with 6 ml of T cell growth medium and transferred to 32°C. Viral supernatants were harvested 48 h posttransfection, cleared by centrifugation at 2500 rpm for 5 min, and either stored in aliquots at −80°C or used to infect cells. The pREV Tet-off viral titer was determined by selecting neomycin-resistant colonies in NIH 3T3 cells and adjusted to a final concentration of 5–8 × 10^6 infectious particles/ml. Although a quantitatively exact titer could not be obtained for the DN/I-κBα viral stocks, we used supernatants from 293-Ampho cultures that were >70% positive for GFP expression.

Assessment of the functionality of the retroviral supernatants

tTA and DN/I-κBα encoding viral supernatants were tested in 293-T cells and Jurkat lymphoblastoid cells. The 293-T epithelial cells were seeded in six-well plates to ~70% confluency and sequentially infected with the pREV and Hermes viral supernatants. Briefly, 1 ml of each supernatant was diluted 1/1 with complete DMEM containing 4 μg/ml of polybrene and added to the cells. The virus was centrifuged onto the cells at 2200 rpm for 90 min at 37°C. Cells were rested for 6 h between infections. Infected cells were grown in the absence or presence of 5 μg/ml tet. The infection efficiency of 293-T cells was about 60%, as determined by GFP expression. Jurkat cell infection followed the same guidelines, with slight modification. Briefly, 2 × 10^5 Jurkat cells were mixed with 1 ml of each viral supernatant, diluted 1/1 in RPMI containing 4 μg/ml of polybrene, transferred to 24-well plates, and centrifuged at 2200 rpm for 90 min. The infection efficiency of Jurkat cells was about 40%, as assessed by flow cytometry. An emission setting of 488 nm was used to detect GFP.

Transduction of primary CD4$^+$ T cells

Primary CD4$^+$ T cells were amplified by CD3/CD28 costimulation, as described above. Three days postamplification, magnetic beads were removed and cells were transferred to 24-well plates coated with Retronectin (Takara Biomedicals) and incubated for 2 h. A total of 1 ml of viral supernatant, diluted 1/1 in T cell-conditioned medium, was added to cells and further centrifuged at 2200 rpm for 90 min. Cells were sequentially treated with the viral supernatants, using three rounds of infection over a 72-h time period in the presence of 5 μg/ml tet and 200 IU/ml of IL-2. On the fourth day postinfection, cells were washed three times in T cell culture medium, and incubated in the presence or absence of 5 μg/ml tet. The percentage of infected T cells was analyzed by flow cytometry, as described above.

Gene expression array

Total cytoplasmic RNA was isolated as described (30). The RNA pellet was resuspended in water and quantitated by absorbance at 260 nm. Approximately 200 μg of cytoplasmic RNA was used to isolate poly(A) RNA using Oligotex resin, as per the manufacturer's instructions (Qiagen, Valencia, CA). Poly(A) RNA was eluted with 2 × 40 μl elution buffer, and the entire solution was quantitated at 260 nm. Typical recovery was 1.5–3 μg. The poly(A) RNA was precipitated with 95% ethanol in the presence of 0.3 M sodium acetate, pH 5.2, and 4 μg of glycogen. The pellet was washed with 75% ethanol, dried, and resuspended in water to a concentration of 0.5 μg/ml poly(A) RNA. The poly(A) RNA (1.5 μg) was used to produce a complex cDNA probe for screening of the Atlas Human cDNA Expression Array (Clontech), as described in the manufacturer's protocol. The gene filters were sealed in plastic and the image data collected with a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Spot labeling, hit picking, and densitometric analysis were performed using a series of Excel-based macros developed at Signal Pharmaceuticals (San Diego, CA).

Apoptosis assays

Apoptosis in primary CD4$^+$, HuT-78, and Jurkat cells was assessed by dual color annexin V/PI staining. Apoptosis was assessed in primary T cells 48 h after removal of tet from retrovirus-infected cells or after incubation for 16 h in the presence of sodium salicylate, prostaglandin PGA_1, PGB_2, and $15dPGJ_2$ at the indicated concentrations. Apoptosis was also assessed in all three cell types 16 h after the introduction of 100 ng/ml anti-Fas mAb, 50 ng/ml TNF-α (in the presence of 10 μg/ml cycloheximide), or 1 μM staurosporine. Cells were incubated with 1 μl annexin V plus 10 μl PI in 500 μl binding buffer for 15 min, as recommended by the manufacturer. Flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA), equipped with a single 488-nm Argon laser. Annexin V-FITC and PI were analyzed at emission settings of 535 and 575 nm, respectively. Dual color staining for mitochondrial membrane potential ($\Delta\varphi_m$) and superoxide production was performed by $DiOC_6(3)$ and HE staining (31). Briefly, 1 × 10^6 cells were incubated with 20 nM $DiOC_6$ and 2 μM HE for 30 min at 37°C in the dark. Cells were subjected to flow

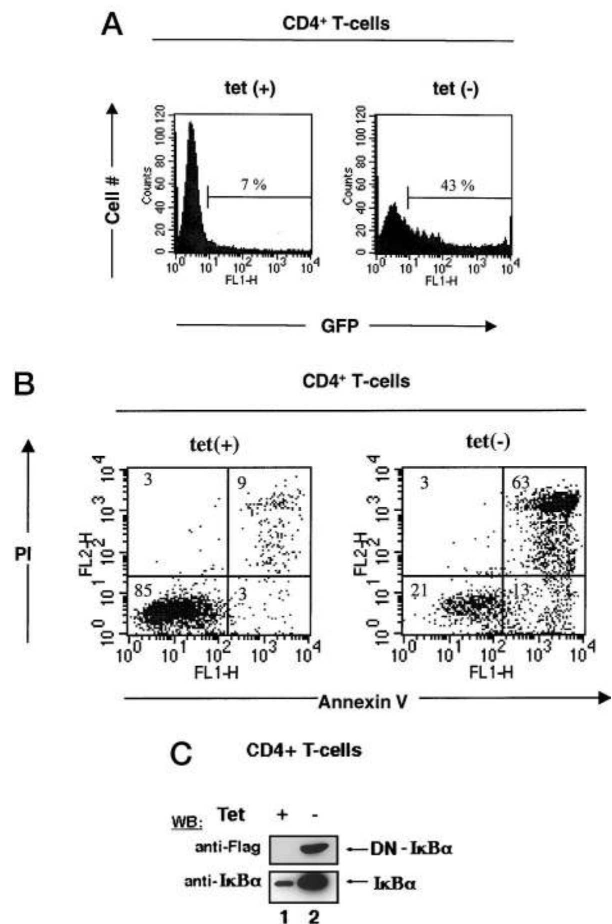

FIGURE 2. Primary human CD4$^+$ T cells transduced by the DN/I-κBα retrovirus undergo apoptosis. *A*, Proliferating CD4$^+$ blasts were transduced with tTA and DN/I-κBα retroviruses in the presence or absence of 5 μg/ml tet, as described in *Materials and Methods*. Two days postinfection, cells were harvested, washed, and examined for GFP expression by flow cytometry. *B*, Dual color annexin V/PI staining to show apoptotic cells in the same populations as determined by flow cytometry (31). *C*, Western blot analysis showing DN/I-κBα expression in the same populations as in *A*. The *top panel* shows blotting with an anti-Flag Ab, as described in Fig. 1*B*. The *bottom panel* shows blotting of similar lysates probed with anti-I-κBα Abs to compare the levels of recombinant and endogenous I-κBα. These experiments were reproduced twice.

cytometry using emission settings of 501 nm for $DiOC_6(3)$ and 605 nM for HE.

EMSA

Gel-shift analysis of nuclear extracts from primary CD4$^+$ T cells and T cell lines was performed as previously described (5). A total of 6 × 10^7 resting primary CD4$^+$ T cells or Jurkat was left untreated or incubated in the presence of 20 mM sodium salicylate for 2 h before stimulation for 30 min with anti-CD3 mAb or a combination of anti-CD3 plus anti-CD28 mAbs, secondarily cross-linked with 187.1 mAb. Cells were immediately washed and processed for extraction of nuclear extracts. A double-stranded oligonucleotide containing the putative NF-κB binding site at positions −840 to −848 of the murine Bcl-x_L promoter (26) (5′-cgatAAAGGGACTTCCAA Gat-3′ and 3′-taTTTCCCTGAAGGTTCtagc-5′) was end labeled with the Klenow polymerase in the presence of [^{32}P]GTP and [^{32}P]dCTP. DNA binding was performed at room temperature for 20 min in a final volume of 20 μl, which contained 5 μg of nuclear extract, 5 × 10^4 cpm labeled oligonucleotide, and binding buffer containing 100 μg/ml of poly(dI-dC) as nonspecific competitor. Shift complexes were resolved on nondenaturing 5% polyacrylamide gel in 0.5× TBE. Specific competition was performed with a 50-fold excess of the unlabeled probe as well as Abs to p50 and p65, as previously described (26).

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Table I. *Apoptosis-associated CD3/CD28-inducible early response genes in $CD4^+$ T cells*[a]

GenBank Accession No.	Description	Average Signal Intensity (fold increase)[b]		
		Control	Anti-CD3	Anti-CD3 + anti-CD28
M32315	TNF receptor 2	6,365	5,216 (0.8)	4,894 (0.8)
X01394	**TNF-α**	**1,538**	**36,494 (23.7)**	**140,506 (91)**
L41690	TRADD	1,538	4,081 (2.6)	1,753 (1.2)
U21092	CD40R-associated factor	3,622	1,337 (0.4)	3,397 (0.9)
L07414	CD40L	NS	NS	NS
L11015	Lymphotoxin-β	30,965	38,754 (1.3)	29,111 (0.9)
Y09392	WSL-LR (Apo-3)	15,198	7,957 (0.5)	10,300 (0.7)
U57059	TRAIL/Apo-2	NS	NS	NS
D38122	**Fas ligand (CD95L)**	**1,538**	**1,062 (0.7)**	**4,205 (2.7)**
M67454	Fas (CD95)	NS	NS	NS
L08096	**CD27 ligand (CD70)**	**1,538**	**1,568 (1.0)**	**4,181 (2.7)**
Z23115	**Bcl-x**	**1,538**	**1,775 (1.2)**	**12,344 (8.0)**
M14745	Bcl-2	NS	NS	NS
U59747	Bcl-w	NS	NS	NS
U45878	cIAP1	NS	NS	NS
U45879	cIAP2	NS	NS	NS
L22475	Bas (β)	6,311	3,264 (0.5)	8,835 (1.4)
D15057	DAD-1 (defender against cell death-1)	26,266	13,067 (0.5)	7,523 (0.3)
U29680	Blf-1 (A_1)	NS	NS	NS
U13737	CPP32 (caspase 3)	10,311	1,659 (0.2)	855 (0.1)
U28014	ICH-2 (caspase 4)	3,767	7,675 (2.0)	855 (0.2)
U20537	Mch-2 (caspase 6)	NS	NS	NS
U39613	Mch-3 (ICE-LAP3)	NS	NS	NS
U60519	Mch-4	NS	NS	NS
U60520	Mch-5 (caspase 8)	NS	NS	NS
L16785	Nucleoside diphoshate kinase	98,984	61,006 (0.6)	54,514 (0.6)
S90469	**NADPH-Cyt P450 reductase**	**1,538**	**522 (0.3)**	**4,013 (2.6)**
L29511	Grb2	15,952	5,362 (0.3)	8,960 (0.6)
X08020	GST (M4)	1,538	5,650 (3.7)	6,759 (4.4)
X86779	FAST kinase	3,057	2,697 (0.9)	2,391 (0.8)

[a] Methodology: Primary human $CD4^+$ T-lymphocytes were grown up in large quantities through stimulation with anti-$CD3^+$ anti-CD28 mAb immobilized on magnetic beads. After completion of their growth cycle, cells were rested for 48 h and 1×10^8 cells treated for 1 h with anti-CD3 or anti-$CD3^+$ anti-CD28 mAb, secondarily cross-linked with 187.1 mAb, as described in *Materials and Methods*. RNA was extracted and reversed transcribed to produce a cDNA probe for screening of the Atlas Human cDNA Expression Array (Clontech). Processing and densitometric analysis of the gene filters were done as described in *Materials and Methods*. NS, no signal. Bold items denote those genes products which increase >2-fold upon CD3 + CD28 stimulation.

[b] Average of two separate measurements in which the SEM of the relative signaling intensity varied ≤10%. Fold increase was calculated by using the control value as denominator.

Immune complex kinase assays

A total of 4×10^7 resting $CD4^+$ T cells was treated with 20 mM sodium salicylate for 2 h before stimulation for 30 min with 2 μg/ml anti-CD3 or a combination of 2 μg/ml anti-CD3 + 2 μg/ml anti-CD28 mAb, secondarily cross-linked with 10 μg/ml mAb 187.1. Stimulation with PMA (100 nM) plus ionomycin (1 μg/ml) was used as a positive control. After cell lysates were precleared with protein A-Sepharose beads, 200 μg lysate was treated with 2 μg anti-IKKβ absorbed onto protein A-Sepharose beads for 2 h. Immune complexes were washed and equilibrated in kinase buffer, as described (32). Kinase reactions were initiated by the addition of 10 μCi [γ-^{32}P]ATP and 3 μg GST-I-κBα (1–54) substrate. The reaction was conducted for 30 min at 30°C. Products were analyzed on SDS-PAGE and detected by autoradiography.

Luciferase assays

A 1.2-kb Bcl-x_L promoter element, cloned upstream of a luciferase gene (pGL2 vector), was kindly provided by Dr. G. Nunez (University of Michigan, Ann Arbor, MI) (33), while wild-type (pGL848) and NF-κB mutant (pGL$_2$κBM) versions of a 848-bp luciferase reporter were a generous gift from Dr. Mari Kannagai (Tokyo Medical and Dental University, Japan) (26). A total of 12 μg of the indicated reporter gene constructs was transiently transfected into 10^7 Jurkat cells in the presence or absence of indicated amounts of IKKβ (34). The cells were rested for 24 h and then stimulated for 6 h with a combination of 2 μg/ml anti-CD3 + 2 μg/ml anti-CD28 mAb, secondarily cross-linked with 187.1 mAb. Where indicated, transfectants were incubated in the presence of 20 mM sodium salicylate or 20 μM 15dPGJ$_2$ prior to stimulation. Control stimulation was with 100 nM PMA + 1 μg/ml ionomycin. The cells were lysed in luciferase buffer (Analytical Luminescence, Ann Arbor, MI), and luciferase activity was measured in 50 μg of lysate in a Monolight 2010 Luminometer (Analytical Luminescence). Transfection efficiency was monitored by cotransfection of a β-galactosidase plasmid (CMV-β-gal); β-galactosidase activity was used for adjusting luciferase values between different transfected cell populations.

Western blot analysis

A total of 50 μg of cellular lysate was separated by SDS-PAGE and transferred to nitrocellulose membranes, as previously described (5). Membranes were sequentially overlaid with specific primary and secondary Abs conjugated to HRP, as indicated in the figure legends. Products were detected by enhanced chemiluminescence (ECL).

Results

Tet-regulated expression of a dominant-interfering I-κBα mutant leads to apoptosis in primary human $CD4^+$ T cells

Coculture of primary human $CD4^+$ lymphocytes with anti-CD3 plus anti-CD28 mAb coimmobilized on beads induces a prolonged (12–16 days) proliferative cycle during which the majority of $CD4^+$ T cells are protected from activation-induced cell death by a signal imparted via the CD28 receptor (25, 28). This signal may involve the NF-κB cascade, which is activated in a synergistic fashion by TCR and CD28 costimulation (5). We asked whether the introduction of a degradation-resistant I-κBα mutant will affect the survival of CD3/CD28-stimulated human $CD4^+$ lymphocytes.

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Table II. *Nonapoptosis-related CD3/CD28-inducible early response genes in human CD4$^+$ lymphocytes*

Gene	GenBank Accession No.	Fold Induction[b]	AP-1[c]	NF-κB[c]
Transcription factors				
ETR-103 transcription factor	M62829	69		
c-Jun	J04111	50	+	
Fra-1	X16707	4.2	+	
NF-Atc	U09579	12		
ETR-101 transcription factor	M62831	5.2		
c-Myc	V00568	3.5		+
Tristetraproline (zinc finger TF)	M92843	2.6		
GATA-3	X55122	2.1		
Enzymes				
NADH unibquinone oxidoreductase	M33374	6.6		+
CDK inhibitor 1A (p21)	U09579	6.3		
Cytokines/chemokines				
TNF-α	X01394	91	+	+
MIP-1α	M23452	7.5		+
GM-CSF	M11220	4.8		+
IL-13	L06801	3.4		
Leukemia inhibitory factor	X13967	3.3		
IL-2	A14844	3.1	+	+
RANTES Receptors	M21121	2.2	+	+
NAK-1 (Nur77)	L13740	9.3		+
Neuromedin B receptor	M73482	6.0		
ICAM-1	J03132	5.2		+
IL-2 receptor γ chain	M26062	3.5		
TGF-β-receptor type III	L07594	3.0		
Stress response				
HSP-60	M34664	5.4		
HSP-70	M11717	5.1		
Other				
Proliferation-associated protein	X67951	3.6		

[a] Experimental approach was as for Table I, including the use of the Atlas Human cDNA Expression Array (Clontech).
[b] Fold induction was calculated as CD3/CD28-induced vs control signaling intensity. Only gene products with a value >2.0 are shown.
[c] Confirmed presence of AP-1 and NF-κB regulatory sites in the promoters of these genes.

That goal was accomplished by using DN/I-κBα expression by a tet-regulated system that uses a combination of the pREV Tet-off and Hermes HRIgfp retroviruses. The former virus encodes for a tTA protein, while the latter expresses a copy of I-κBα(32A/36A) downstream a heptamerized tet operator (Fig. 1*A*) (29). The Hermes virus also coexpresses GFP, which allows the tracking of infected cells by fluorescence microscopy or flow cytometry (Fig. 1*A*). The efficiency of these retroviruses was demonstrated in 293 and Jurkat cells, in which tet removal from the culture medium could be seen to induce Flag-tagged DN/I-κBα (Fig. 1*B*) and GFP (not shown) expression. These infection conditions were used to develop optimal conditions for infection of primary lymphocytes.

In experiments in which these retroviruses were used to infect CD3/CD28-stimulated CD4$^+$ blasts, we observed GFP expression in >40% of the tet$^{(-)}$ cells (Fig. 2*A*). In contrast, only 7% of tet$^{(+)}$ cells expressed GFP (Fig. 2*A*). When the same populations were used to assess cell viability by flow cytometry, we could demonstrate an increase from 9 to 63% in the percentage of annexin V$^+$/PI$^+$ cells in the tet$^{(-)}$ as compared with tet$^{(+)}$ population (Fig. 2*B*). DN/I-κBα expression in tet$^{(-)}$ cells was confirmed by anti-Flag immunoblotting (Fig. 2*C*, *top panel*). Examination of similar lysates with an antiserum that recognizes I-κBα protein revealed that DN/I-κBα was overexpressed compared with endogenous I-κBα levels (Fig. 2*C*, *bottom panel*). Taken together, these data indicate that interference in the NF-κB pathway by a degradation-resistant I-κBα mutant negates the anti-apoptotic effect of the CD28 receptor in primary human CD4$^+$ lymphocytes.

NF-κB activity is linked to Bcl-x_L expression and cellular survival in primary CD4$^+$ T lymphocytes

To determine which genes function as early response elements during CD3/CD28 costimulation, we used the human Atlas (Clontech) cDNA expression array, which includes an apoptosis-related panel. Reverse-transcribed RNA from CD4$^+$ lymphocytes, treated for 1 h with the anti-CD3 + anti-CD28 mAb combination or anti-CD3 mAb alone, was used for this analysis. While there was no early message expression for anti-apoptotic proteins such as cIAP1, cIAP2, cIAPx, Bfl-1, and Bcl-2, CD3 + CD28 stimulation induced Bcl-x_L expression (Table I). In contrast, CD3 ligation alone had a negligible effect on Bcl-x_L expression (Table I). All of the aforementioned genes have been linked to NF-κB activation (26, 27, 35–38). Interestingly, CD3 + CD28 costimulation also up-regulated expression of a number of pro-apoptotic genes, including TNF-α and Fas ligand (Table I). CD28 costimulation also induced nonapoptosis-related genes, belonging to the transcription factor, enzyme, cytokine/chemokine, receptor, and stress response categories (Table II). The expression of some of these genes may involve activation of their promoter by AP-1 and NF-κB response elements, which are CD28 inducible (Table II).

The Bcl-x_L gene product has a clear link to the anti-apoptotic effects of the CD28 receptor (24, 39, 40). Moreover, a number of investigators have recently identified functional NF-κB response elements in the human and murine Bcl-x_L promoters, which may play a role in expression of the protein in T lymphocytes (26, 27).

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We confirmed at protein level that Bcl-x_L expression in primary human $CD4^+$ lymphocytes is linked to CD28 costimulation (Fig. 3*A*). While CD3 + CD28 costimulation induced Bcl-x_L expression in $CD4^+$ T cells, anti-CD3 mAb alone had little effect (Fig. 3*A*). In contrast, cIAP2 was constitutively expressed in these cells, and its relative abundance was not further increased during cellular stimulation (Fig. 3*B*). The same was true for cIAP2 expression in the Jurkat T cell line (Fig. 3*B*).

To explore the link between Bcl-x_L expression and NF-κB activation, we made use of the inhibitory effects of Na^+-salicylate on IKKβ activation (41). This drug effectively suppressed IKK activation in $CD4^+$ lymphocytes during CD3 + CD28 costimulation (Fig. 3*C*), and also had an inhibitory effect on Bcl-x_L expression during immunoblotting (Fig. 3*A*, *lane 6*). Although it has been suggested that salicylate can induce apoptosis through the activation of the $p38^{MAPK}$ cascade (42), we could not demonstrate that a specific inhibitor of that cascade (SB203580) exerted any effect on Bcl-x_L expression or that it could negate the inhibitory effects of Na^+-salicylate (Fig. 3*D*). A second chemical inhibitor of the NF-κB cascade in T lymphocytes, pyrrolidine-dithiocarbamate (43), also interfered in Bcl-x_L expression in primary human $CD4^+$ T cells (not shown).

In the next of experiments, we asked whether Na^+-salicylate affected $CD4^+$ cell viability, and demonstrated a dose-dependent increase in apoptosis in CD3 + CD28-treated cells (Fig. 4*A*). To confirm the specificity of IKKβ in this cellular effect, we also examined the effect of the cyclopentenone PGs, $15dPGJ_2$ and PGA_1, which have recently been shown to directly inhibit the IKKβ subunit of the IKK complex in Jurkat cells (44). As shown in Fig. 4*B*, treatment of proliferating $CD4^+$ T cells with these cyclopentenone PGs induced apoptosis in a dose-dependent fashion. In contrast, PGB_2, which does not inhibit IKKβ, had no effect on $CD4^+$ T cell survival (Fig. 4*B*). Collectively, these data indicate that IKKβ activity is essential for the survival of primary human $CD4^+$ T cells.

Activation of the Bcl-x_L promoter via a NF-κB response element in T lymphocytes

To show more directly activation of the Bcl-x_L gene during CD28 costimulation, we used a 1.2-kb fragment of the murine promoter linked to a luciferase reporter (Bcl-x_L-Luc) for transfection into the Jurkat T cell line (33). Although Jurkat cells failed to show endogenous Bcl-x_L expression during CD28 costimulation (see Fig. 5*A*), CD3 + CD28 ligation had a stimulatory effect on reporter gene activity (Fig. 5*A*). This response was enhanced by the addition of PMA or cotransfection of IKKβ cDNA (Fig. 5*A*). Please notice that exogenously expressed IKKβ (Fig. 5*B*) is constitutively active in Jurkat cells (4, 5), which may explain the 2.1-fold increase in basal luciferase activity in these transfected cells (Fig. 5*A*). Moreover, the costimulatory effects of CD3 + CD28 on the Bcl-x_L promoter were inhibited by treating the cells with the pharmacological inhibitors, Na^+-salicylate and PG $15dPGJ_2$, confirming that IKKβ activity plays a role in Bcl-x_L promoter activity (Fig. 5*C*).

An NF-κB-binding motif (GGGACTTCC), similar to the NF-κB element in the immunoglobin promoter, has been identified in the murine Bcl-x_L promoter (26). Mutation of this site, located at bp −848 to −840 from the start site in an 848-bp promoter, significantly decreased the promoter response to NF-κB activation in a murine CTLL cell line (26). When used in Jurkat cells, the mutant promoter could not be induced by CD3 + CD28 costimulation, and did not respond to ectopic expression of IKKβ either (Fig. 5*D*). Moreover, we demonstrated that nuclear extracts from Jurkat cells contain CD3 + CD28-inducible factor(s) that binds to a labeled oligonucleotide probe corresponding to that NF-κB response element (Fig. 6*A*). Complex formation was competed by a

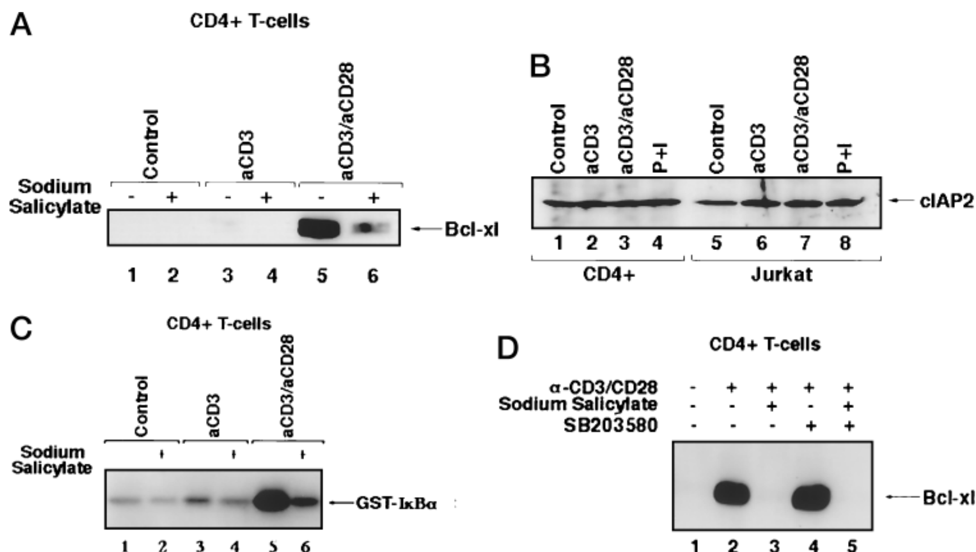

FIGURE 3. Sodium salicylate interferes with IKK activity and Bcl-x_L expression in $CD4^+$ T cells. *A*, Western blot analysis of Bcl-x_L expression in Na^+-salicylate-treated $CD4^+$ cells. A total of 10^7 resting cells was either left untreated or stimulated with anti-CD3 mAb or a combination of anti-CD3 + anti-CD28 mAb bound to magnetic beads in the absence or presence of 20 mM sodium salicylate for 16 h. A total of 50 μg lysate was examined for Bcl-x_L expression using anti-Bcl-x_L mAb (1:1000), followed by sheep anti-mouse HRP (1:2000). *B*, Western blot analysis of $CD4^+$ and Jurkat T cell lysate for cIAP2 expression, using a goat anti-cIAP2 Ab (1:1000), followed by rabbit anti-goat HRP (1:2000). Jurkat and primary $CD4^+$ T cells were stimulated as in *A*. *C*, Sodium salicylate blocks IKK activity. Resting primary human $CD4^+$ T cells were either left untreated or exposed to 20 mM sodium salicylate for 2 h before stimulation for 30 min with anti-CD3 or anti-CD3 plus anti-CD28 mAb (2 μg/ml each), secondarily cross-linked with 10 μg/ml 187.1 mAb. Cells were lysed and IKK complexes were immunoprecipitated with 2 μg of an anti-IKKβ antiserum bound to protein A-Sepharose beads. Kinase assays were performed using the substrate GST/I-κBα(1–54), as described in *Materials and Methods*. Products were analyzed by SDS-PAGE and autoradiography. The arrow points to phosphorylated GST/I-κBα. *D*, Western blot analysis for Bcl-x_L expression using CD3 + CD28 ligation in the absence or presence of 10 μM SB203580 and/or 20 mM sodium salicylate.

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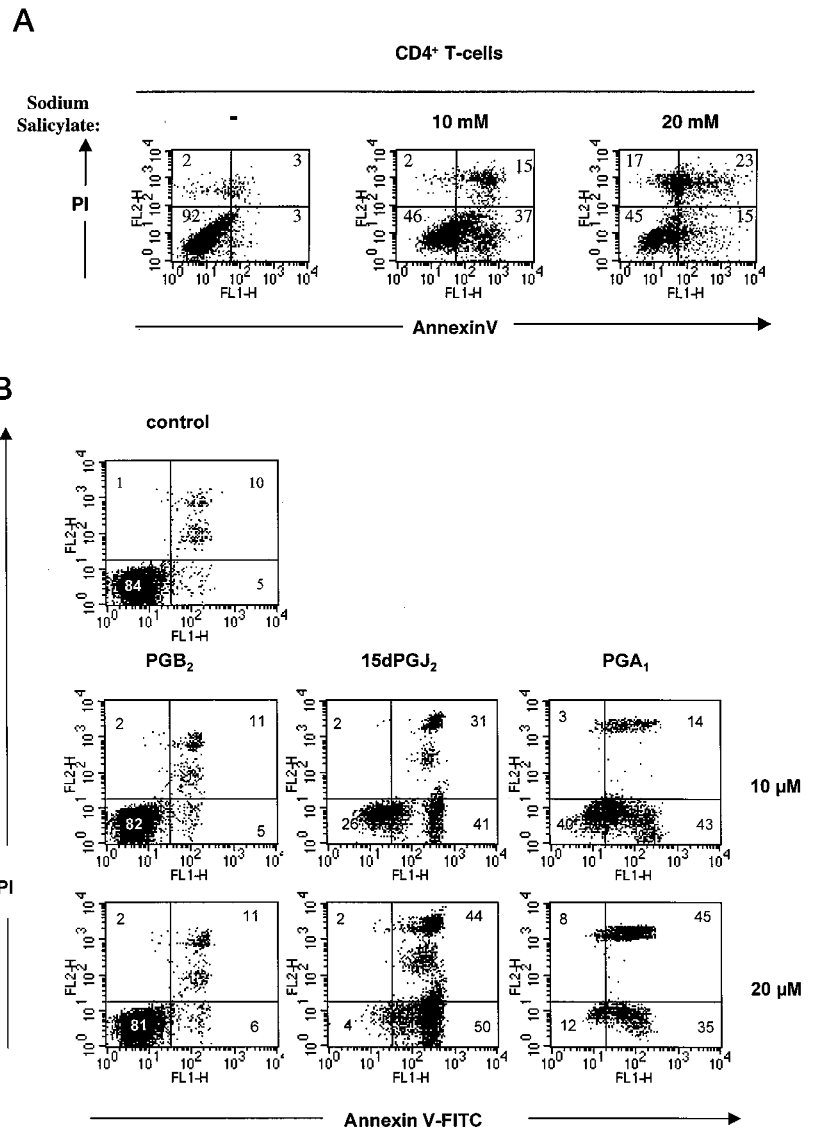

FIGURE 4. Dual color annexin V/PI staining to assess induction of apoptosis in CD4$^+$ T cells treated with pharmacological inhibitors of IKKβ. *A*, Primary CD4$^+$ T cells were stimulated with anti-CD3 + anti-CD28 Abs coupled to magnetic beads. On day 3, proliferating T cells were treated for 16 h with indicated concentrations of Na$^+$-salicylate (*A*) or prostaglandins PGB$_2$, 15dPGJ$_2$, and PGA$_1$ (*B*). After staining with annexin V and PI, cells were analyzed by flow cytometry. Similar results with Na$^+$-salicylate were obtained with CD4$^+$ T cells from two additional donors.

50-fold excess of the cold probe (Fig. 6*A*, *lane 4*), and the complex was partially supershifted with Abs to p50 and p65, as previously described (26). It is interesting that P+I treatment, which leads to IKK activation, induced the same shift complex (Fig. 6*B*, *lane 5*). Similar effects were seen in CD4$^+$ T cells during treatment with anti-CD3 + anti-CD28 or P+I (Fig. 6*B*, *lanes 3* and *4*). Na$^+$-salicylate interfered in the generation of these shift complexes in Jurkat as well as CD4$^+$ T cells (Fig. 6, *A* and *B*). Similarly, the cyclopentenone PGA$_1$ inhibited PMA-induced NF-κB shift complexes and IKK activation in Jurkat cells (44).

Bcl-x$_L$ expression in CD4$^+$ blasts and HuT-78 cells is associated with different apoptosis profiles than in non-Bcl-x$_L$-expressing Jurkat cells

While the anti-apoptotic effects of the NF-κB cascade cover a wide range of stimuli, the role of Bcl-x$_L$ to diverse stimuli has not been studied in any depth in primary human T cells. To perform those studies, we used Bcl-x$_L$-expressing CD4$^+$ blasts to look at the effect of Fas, TNF-α, and staurosporine. For comparison, we included a Jurkat cell clone, which does not express detectable levels of endogenous Bcl-x$_L$ (Fig. 7*A*), as well as HuT-78, which shows constitutive Bcl-x$_L$ expression (Fig. 7*A*). Interestingly, this constitutive Bcl-x$_L$ expression in HuT-78 cells occurs in parallel with constitutive NF-κB activation (45). That fact is demonstrated by the ability of nuclear extracts from unstimulated HuT-78 cells to generate a shift complex with a probe representative of the NF-κB site in the Bcl-x$_L$ promoter (Fig. 7*B*). Cellular stimulation with CD3 + CD28 mAb or P+I did not increase the relative abundance of this shift complex (not shown).

In additional experiments, proliferating CD4$^+$ blasts, HuT-78, and Jurkat cells were exposed to anti-Fas mAb, TNF-α, and staurosporine for 16 h, and subjected to two-color annexin V/PI staining. While these stimuli readily induced apoptosis in Jurkat cells, there was a comparatively minor effect in CD4$^+$ blasts and HuT-78 cells (Fig. 8*A*). This is compatible with the wide range of anti-apoptotic effects of Bcl-x$_L$ in T cells. Included among those effects is regulation of the mitochondrial permeability transition pore complex, which has an effect on $\Delta\varphi_m$ as well as release of apoptogenic factors from the mitochondrium (46, 47). The drop in $\Delta\varphi_m$ during mitochondrial perturbation can be assessed in a flow cytometer with the fluorochrome, DiOC$_6$(3). We therefore looked at the decrease in DiOC$_6$(3) fluorescence in HuT-78 vs Jurkat cells, and combined that with measurement of superoxide (O_2^-) generation, as determined by HE staining (31). O_2^- generation is a reflection of damage to the mitochondrial inner membrane, which disrupts electron transfer between complexes I and III (31). This

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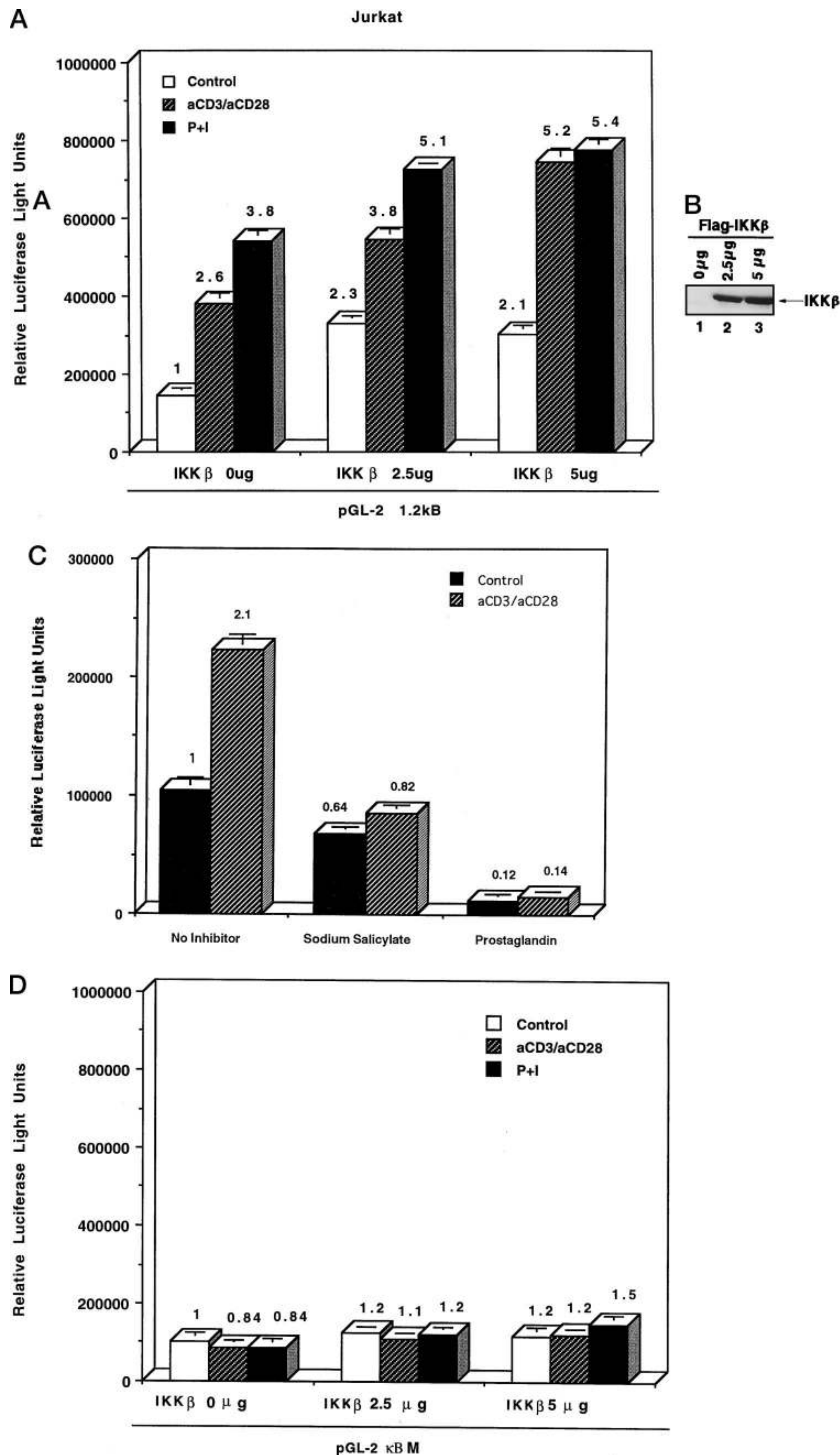

FIGURE 5. CD3 + CD28 ligation activates the murine Bcl-x_L promoter in Jurkat cells. *A*, Jurkat T cells were transfected with 12 μg of the 1.2-kb Bcl-x_L-Luc reporter in the absence or presence of 2.5 or 5 μg IKKβ. After resting for 24 h, cells were stimulated with anti-CD3 plus anti-CD28 mAb, secondarily cross-linked with 187.1, or PMA (100 nM) + ionomycin (1 μg/ml) for 6 h. Cells were lysed and examined for luciferase activity, as described in *Materials and Methods*. *B*, Anti-Flag immunoblotting to show IKKβ expression in *A*. *C*, Jurkat T cells were transfected with 12 μg of the 1.2-kb Bcl-x_L-Luc reporter. After resting for 24 h, cells were either left untreated or treated with 20 mM of sodium salicylate or 20 μM PG 15dPGJ$_2$ for 1 h before stimulation as in Fig. 4*A*. *D*, Similar to Fig. 4*A*, except a Bcl-x_L promoter with mutated NF-κB binding site was used as a reporter.

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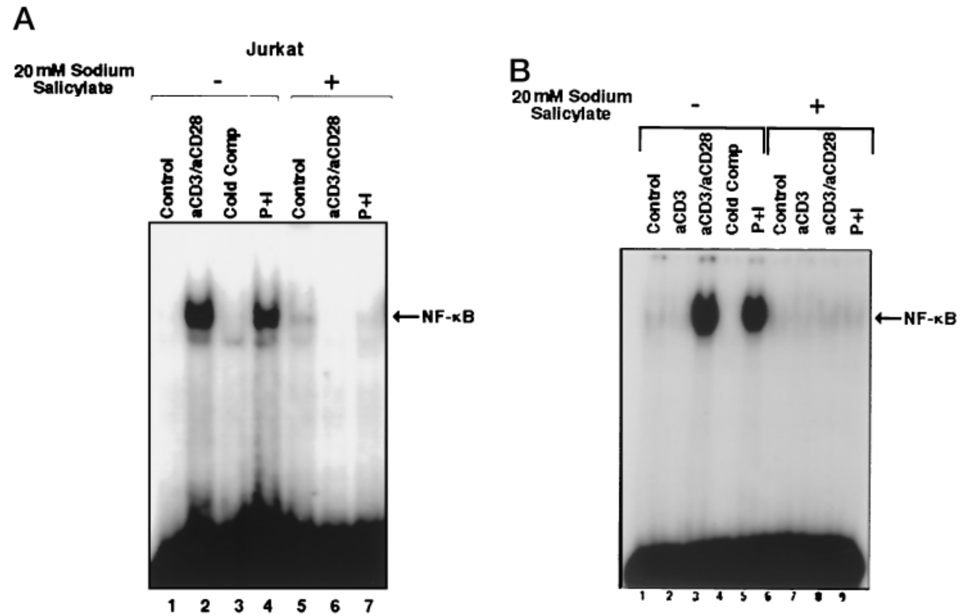

FIGURE 6. EMSA analysis of Jurkat and primary $CD4^+$ T cell nuclear proteins. *A*, Nuclear extracts were prepared from resting or stimulated cells, which were incubated in the presence or absence of 20 mM Na^+-salicylate, as indicated. Cells were stimulated with anti-CD3 + anti-CD28 mAb or P+I for 30 min. These extracts were incubated with 1×10^5 cpm of ^{32}P-labeled oligonucleotide corresponding to the NF-κB binding site at position −840 to −848 of the murine promoter. Cold competition was performed with a 50-fold excess of unlabeled oligonucleotide. *B*, Similar EMSA as described in *A*, except primary $CD4^+$ T cell nuclear extracts were used. Results were reproduced twice.

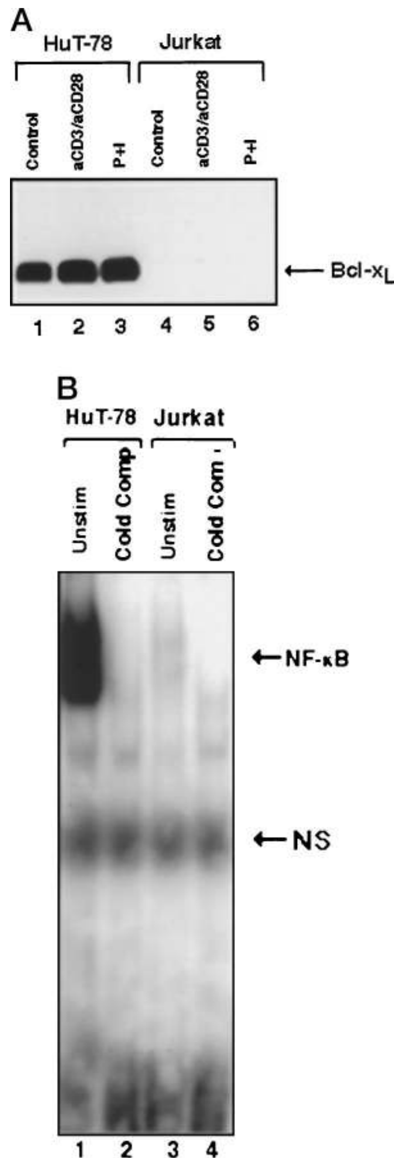

FIGURE 7. Bcl-x_L expression in HuT-78 and Jurkat T cells correlates with NF-κB activation. *A*, Western blot analysis of HuT-78 and Jurkat T cells for Bcl-x_L expression. Cellular stimulation and performance of immunoblotting were as described in the legend of Fig. 3*A*. *B*, EMSA on Jurkat and HuT-78 nuclear extracts. Samples were processed as described in the legend of Fig. 4. Similar data were obtained in duplicate experiment.

experiment showed that while staurosporine, anti-Fas, and TNF-α had a considerable effect on $\Delta\varphi_m$ as well as O_2^- generation in Jurkat cells, the same stimuli had only a minor effect on HuT-78 cells (Fig. 8*B*). This suggests that the constitutively active NF-κB cascade and accompanying high levels of Bcl-x_L expression in HuT-78 cells exert important anti-apoptotic effects at mitochondrial level.

Discussion

We show that CD3/CD28 costimulation in primary human $CD4^+$ T cells induces Bcl-x_L expression in parallel with activation of a NF-κB response site in the promoter of that gene. Retroviral-mediated expression of a DN/I-κBα mutant as well as treatment with direct inhibitors of IKKβ activity, namely Na^+-salicylate and cyclopentenone PGs, induced apoptosis in primary $CD4^+$ T cells. Since this suggests that the CD3/CD28-induced NF-κB cascade exerts anti-apoptotic effects in primary T cells via Bcl-x_L expression, we showed that the activation of the promoter of that gene required NF-κB activation and was induced by IKKβ expression in Jurkat T cells. Moreover, HuT-78, a T cell line with constitutive activation of the NF-κB cascade, exhibited high basal levels of Bcl-x_L expression. HuT-78 cells, like proliferating primary $CD4^+$ T cells, have a relatively high resistance to Fas-, TNF-α-, and staurosporine-induced apoptosis compared with a Jurkat cell clone in which Bcl-x_L was not detectable. Therefore, among a range of apoptosis-related proteins induced by CD28 costimulation, Bcl-x_L plays a major role in apoptosis prevention.

Stimulus-induced degradation of inhibitory I-κBα and I-κBβ proteins activates NF-κB transcription factors (6, 7). We have been using Ab-coated beads as surrogate APCs to amplify primary $CD4^+$ T cells in studying the role of NF-κB pathway in proliferation, cell death, and anergy (5, 28). Using a retrovirus-mediated gene transfer approach, we found that interruption of NF-κB pathway in $CD4^+$ T cells with superinhibitory DN/I-κBα inhibited cell

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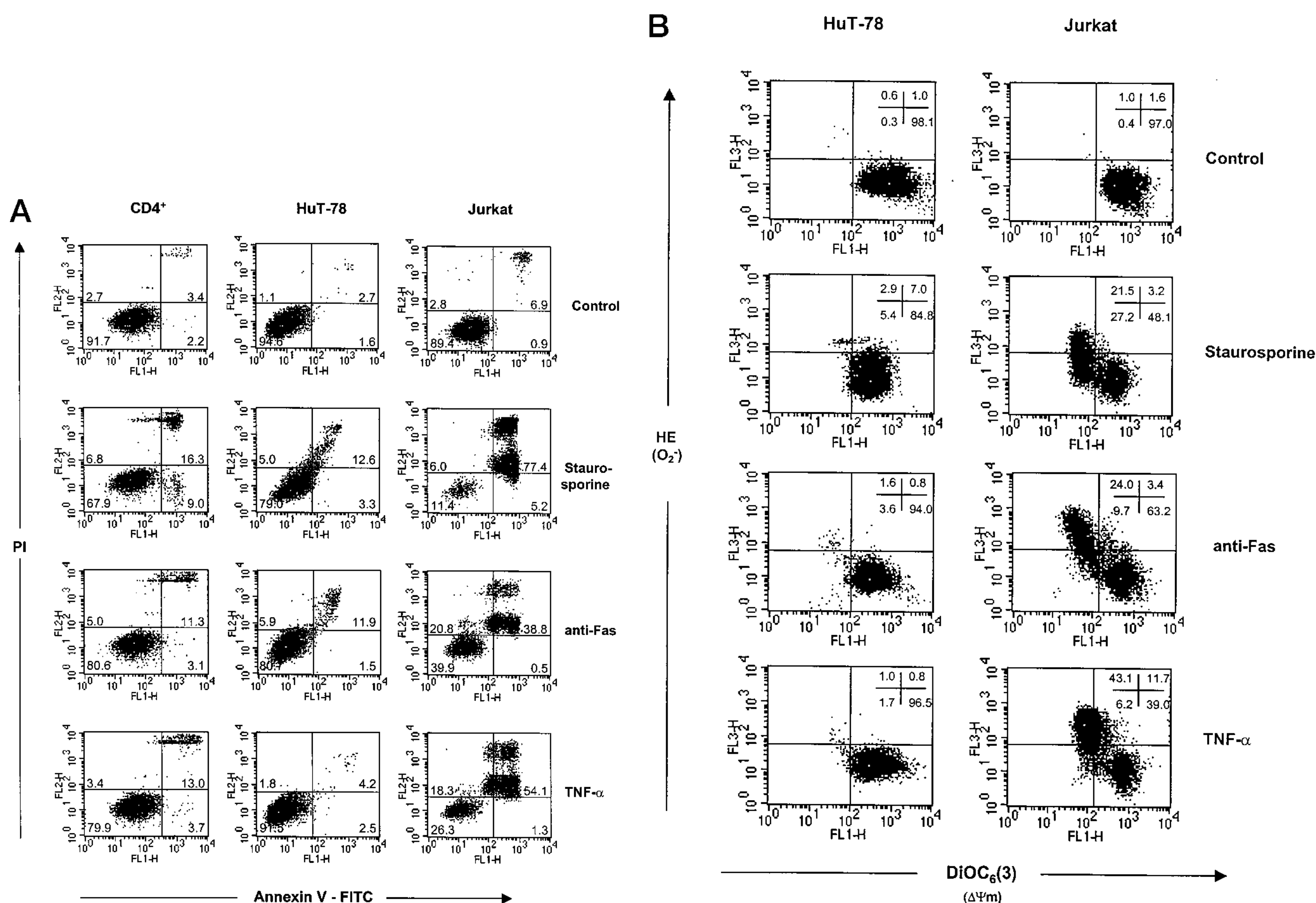

FIGURE 8. Contrasting apoptosis profiles in proliferating $CD4^+$ T cells and HuT-78 compared with Jurkat. *A*, Dual color annexin V/PI flow cytometry to compare apoptosis rates in day 3 $CD4^+$ blasts, HuT-78, and Jurkat cells. A total of 1×10^6 cells was treated with 50 ng/ml TNF-α in the presence of cycloheximide, 100 ng/ml anti-Fas mAb, or 1 μM staurosporine for 16 h. Annexin V/PI staining and flow cytometry were performed as described in Fig. 3*D*. *B*, Dual color $DiOC_6(3)$/HE staining in Jurkat and HuT-78 T cells to assess the effect of above stimuli on mitochondrial responses. Cells were incubated with 20 μM $DiOC_6(3)$ and 2 μM HE for 15 min in the dark before assessment in a flow cytometer. Results were reproduced three times.

proliferation and induced apoptosis (Fig. 2*B*). The prosurvival effect of the NF-κB pathway has been demonstrated in many systems, including T cells (18, 26, 48). For example, Kolenko et al. (48) have demonstrated that pharmacological inhibition of NF-κB pathway in PBLs induces caspase-dependent apoptosis. Moreover, CTLA-4 ligation, which has been shown to inhibit the proliferation and survival of T cells, suppresses CD28-mediated activation of NF-κB by blocking I-κBα phosphorylation (49, 50). Therefore, CD28-mediated NF-κB activation is a prerequisite for the proliferation and survival of mature T cells during ligation of the TCR.

The anti-apoptotic effects of NF-κB have been attributed to induction of pro-survival gene products, including Bcl-2, Bcl-x_L, cIAPs, Al/Bfl-1, IEX-1L, TNFR-associated factor 1 and 2, and superoxide dismutase (26, 27, 35–38). Examination of $CD4^+$ T cells revealed that among the anti-apoptotic proteins regulated by NF-κB, CD3/CD28 stimulation resulted in the induction of Bcl-x_L (Table I). Consistent with previous reports, CD3 + CD28 costimulation also induced expression of the pro-apoptotic Fas ligand (Table I). It is interesting, therefore, that Bcl-x_L has been associated with inhibition of Fas-induced apoptosis in murine T cells and exogenously transfected Jurkat cells (39, 51). Bcl-x_L also has the ability to rescue B cells from Fas-induced apoptosis (27, 39). While interference in Fas-induced cell death may explain the prosurvival effect of Bcl-x_L in CD28-costimulated primary $CD4^+$ T cells (Fig. 8*A*), it is equally possible that this may also represent an interference in TNF-α-induced apoptosis. In this regard, our cDNA arrays showed that CD3/CD28 costimulation also induced TNF-α message expression (Table I). While our studies were not designed to address Bcl-x_L interference in a particular cell death pathway, it is noteworthy that the NF-κB pathway has a known negative regulatory effect on TNF-α-induced apoptosis (1). Whatever the primary stimulus for cell death in $CD4^+$ lymphocytes during inhibition of the NF-κB pathway, CD28-costimulated cells were resistant to TNF-α- and Fas-induced apoptosis (Fig. 8*A*).

Several reports, including ours, have suggested that IKKβ activity is responsible for I-κBα phosphorylation in response to the majority of NF-κB-inducing signals (5, 52). Seminal studies in mice in which the IKKβ locus has been deleted support that notion (53–55). Embryonic cells from IKKβ knockout mice do not activate the NF-κB pathway in response to TNF-α or IL-1 stimulation (53), and die at midgestation due to severe liver apoptosis (55). In this study, we show that treatment of primary $CD4^+$ T cells with Na^+-salicylate, an inhibitor of IKKβ (41), blocks Bcl-x_L expression (Fig. 3*D*) as well as inducing apoptosis (Fig. 3*E*). This notion was further substantiated by the pro-apoptotic effects of recently discovered cyclopentenone IKKβ inhibitors, namely prostaglandins PGA_1 and $15dPGJ_2$ (Fig. 4) (44). The induction of apoptosis by Na^+-salicylate in B cells and myeloid leukemia cells has been associated with activation of caspases (56, 57). It is also interesting that Bcl-x_L has been shown to interact with caspases and interferes with the execution of the death phase (58–60). Therefore, it is plausible that inhibition of IKKβ activity by cyclopentenone PGs

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and Na^+-salicylate is responsible for induction of apoptosis in CD28-costimulated T cells.

Proliferating primary $CD4^+$ and HuT-78 lymphoblastoid T cells resisted death in response to various apoptotic stimuli, including TNF-α, anti-Fas mAb, and staurosporine (Figs. 7 and 8). This resistance to apoptosis was also reflected in the resistance of mitochondrial membrane perturbation in HuT-78 cells (Fig. 8*B*). This is compatible with the regulatory effects of Bcl-x_L on the mitochondrial permeability transition pore (47), but does not rule out other anti-apoptotic effects of this regulatory protein. For instance, Bcl-x_L has recently been demonstrated to interact with Apaf-1 and several caspases (58–60). Thus, heterodimerization with pro-apoptotic proteins and competition with Apaf-1 for binding to caspases may represent additional Bcl-x_L activities in primary $CD4^+$ T cells.

The 3.2-kb stretch of DNA upstream of the Bcl-x_L initiation site contains a variety of different transcription factor binding sites (33). While the contribution of most of these elements in regulating the expression of Bcl-x_L remains unclear, some progress has been made. For instance, signaling through IL-6 pathway, which leads to activation of Stat3 and Janus kinases, has been associated with induction of Bcl-x_L expression (61). Indeed, constitutive activation of Stat3 has been associated with the pathogenesis of multiple myeloma through inhibition of apoptosis (61). Two reports have also identified independent NF-κB binding sites in the Bcl-x_L promoter (26, 27). In one of those studies on the human promoter, two tandem NF-κB binding sites were identified in the human promoter approximately 200 bp upstream of the initiation site (27). In the second study, a NF-κB binding site was identified at ~840 bp upstream of the Bcl-x_L initiation site in the murine promoter (26). The activation of the latter binding element by Tax protein is putatively involved in the ability of CTLL-2 cells to survive in the absence of IL-2 (26). DN/I-κBα as well as mutation of this putative NF-κB site abolished Tax-mediated Bcl-x_L promoter activity and expression (26). While a 600-bp promoter element did not respond to CD3/CD28-mediated costimulation or IKKβ overexpression in our studies (not shown), 1.2-kb and 848-bp Bcl-x_L promoter elements were responsive to these stimuli (Fig. 5*A*). The CD3/CD28-induced activation of the 1.2-kb Bcl-x_L-Luc promoter element was blocked by pharmacological inhibtion of IKKβ (Fig. 5*C*). In addition, mutation of the NF-κB site at position −840 to −848 in the 848-bp reporter rendered it nonresponsive to CD3 + CD28 ligation (Fig. 5*D*). Moreover, CD3 + CD28 stimulation resulted in nuclear localization of NF-κB transcription factors that bound to an oligonucleotide probe representative of that NF-κB binding site (Fig. 6) (26). Nuclear localization and binding of these factors were inhibited in primary $CD4^+$ and Jurkat T cells treated with Na^+-salicylate (Fig. 6). Furthermore, constitutive activation of the NF-κB pathway in untreated HuT-78 T cells was associated with constitutive Bcl-x_L expression and generation of an identical NF-κB shift complex (Fig. 7). These studies indicate that CD3 + CD28-induced activation of NF-κB pathway influences Bcl-x_L expression through this putative binding site. Similar to CD3/CD28 stimulation, Tax-mediated NF-κB activation, which also leads to Bcl-x_L expression, proceeds through IKKβ activation (26, 62).

The data presented indicate that regulation of IKKβ activity in $CD4^+$ T cells and expression of the anti-apoptotic protein Bcl-x_L may coordinate the life span and expansion rate of specific T cell clones when they encounter particular Ags. These findings have important implications in the regulation of the mature T cell compartment and disease induction that may result from dysregulated apoptosis in these cells.

Acknowledgments

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