

Nuclear Factor- κ B/Rel Is Apoptogenic in Cytokine Withdrawal-induced Programmed Cell Death¹

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Abstract

In the complex microenvironment where they evolve, developing cells undergo rapid programmed cell death (PCD) when cytokines that support them become limiting. The transcriptional mechanisms of cytokine-withdrawal apoptosis are poorly understood. In this report, we used early B-lymphocyte tissue culture and transgenic cells to demonstrate that nuclear factor- κ B (NF- κ B) promotes apoptosis during cytokine withdrawal-induced PCD. In the progenitor B lymphocyte model FL5.12, whereas NF- κ B has an antiapoptotic function in response to tumor necrosis factor- α , cytokine withdrawal causes nuclear translocation of NF- κ B/cRel, where it is apoptogenic. Inhibition of NF- κ B activation delays cytokine withdrawal-induced PCD in both FL5.12 and transgenic early B cells. Additionally, reconstituting a bone marrow microenvironment *ex vivo* abrogates the differential apoptotic pattern between control and transgenic early B cells.

Introduction

The ubiquitous Rel/NF- κ B³ proteins, which include RelA (p65), c-Rel, v-Rel, RelB, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p52), are transcription factors that regulate genes by binding to recognition sequences (canonical site, GGGACTTCC) in promoter/enhancer regions. NF- κ B molecules form homo- or heterodimers with each other and remain bound in the cytoplasm in an inactive complex with inhibitory proteins called I κ B. The removal of I κ B exposes the nuclear localization sequence of the NF- κ B complex, thereby mobilizing it for nuclear translocation. The NF- κ B family is able to effect a wide variety of responses in the development and function of the immune system because of the unique characteristics of each member and their combinatorial possibilities (1 and references therein).

NF- κ B factors have been implicated both as activators and repressors of PCD, depending on the stimulus and cell type examined. For example, NF- κ B p50/RelA is protective in a TNF- α model of PCD (2–4). On the other hand, there is evidence that NF- κ B may be involved in promoting PCD. v-rel is cytopathic in murine fibroblasts (5), and if expressed in avian cells, it causes a transforming phenotype. In addition, cRel expression in the avian embryo is correlated with cells undergoing PCD (6). Finally, the anti-inflammatory drug aspirin (sodium salicylate) protects neuronal cells by down-regulation of NF- κ B, thereby implicating this family of factors in the promotion

of cell death during inflammation (7). Taken together, these observations indicate that NF- κ B members can have dramatically different effects during PCD in different cell systems.

PCD after growth factor or cytokine withdrawal is a physiological process that occurs during morphological development, neurogenesis, and lymphopoiesis (Ref. 8 and references therein). In these microenvironments, lack of cytokines and other growth factors causes apoptosis of developing cells. It is estimated that 75% of progenitor and precursor B cells die in the bone marrow because of lack of survival signals (Ref. 9 and references therein). Very little is known about the regulation of PCD during these processes. The evolution of B-lineage cells represents an ideal candidate cell type in which to examine the regulation of physiologically relevant developmental PCD.

To identify and characterize early pathways in factor withdrawal-induced PCD, we selected the nontransformed progenitor B-lymphocytic cell line, FL5.12 (10, 11). In these cells, the NF- κ B member RelA is constitutively present in the nucleus. Between 2 and 8 h after cytokine withdrawal, the major NF- κ B inhibitor, I κ B- α , is degraded and a different NF- κ B member, cRel, is translocated to the nucleus, and cells die by apoptosis. We show that in FL5.12 cells, the stable expression of a transdominant inhibitor of NF- κ B activity, termed I κ B- α Δ N (12), significantly delays death after cytokine withdrawal. In addition, in FL5.12 cells transient overexpression of I κ B- α Δ N delays PCD after cytokine withdrawal, whereas RelA has no effect and cRel precipitates PCD after cytokine withdrawal. Finally, bone marrow-derived B cells from transgenic mice expressing I κ B- α Δ N die more slowly than nontransgenic controls when cultured in the absence of survival factors. This role of NF- κ B in PCD is specific to cytokine withdrawal because when cytokines are provided exogenously, no difference in rates is observed.

Materials and Methods

Cell Culture, Cloning, and Transfections. FL5.12 cells grown as described previously (10, 11) were deprived of cytokine for the indicated times, and the percentage of viability (trypan blue exclusion) and the apoptotic index (as a percentage; ethidium bromide/acridine orange staining) were determined as described (13). FL5.12 cells were transfected with pSFFV-I κ B- α Δ N, stable clones were isolated under G418 selection, and individual I κ B- α Δ N-expressing clones were identified by Western blot analysis. Two independent expressing clones were characterized.

For luciferase assays, parental FL5.12 cells and FL5.12 clones expressing I κ B- α Δ N were transiently transfected with either 10 μ g of 2X κ Btk/Luc (IL-2 receptor- α κ B enhancer) or 10 μ g of Mut-2X κ Btk/Luc (a DNA-binding mutant of the IL-2R α - κ B motif) and 2 μ g of SV40- β gal as an internal transfection control, via the DEAE-trypsin protocol. Forty-eight h post transfection, multiple independent transfectants were pooled, the cytokine was withdrawn by washing the cells three times with PBS, and the cells were resuspended in complete medium without IL-3. The time $t = 0$ was taken as the mid-point between the time when the medium containing cytokine was withdrawn and the cytokine without medium was added. Cells (1×10^6) were removed, processed into cell lysates, and analyzed for luciferase activity according to the manufacturer's instructions (Analytical Bioluminescence).

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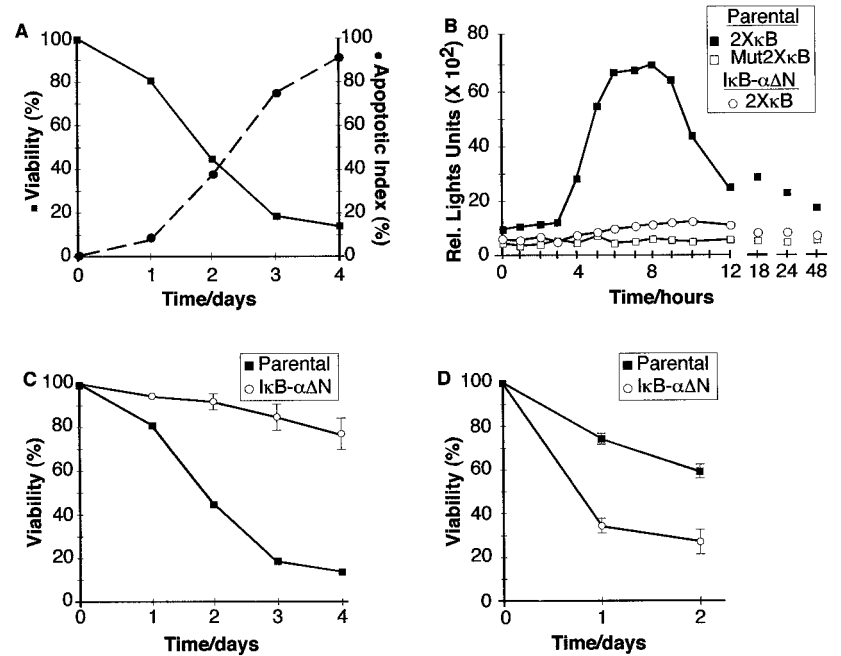
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³ The abbreviations used are: NF- κ B, nuclear factor- κ B; PCD, programmed cell death; TNF- α , tumor necrosis factor- α ; IL, interleukin; GFP, green fluorescent protein.

Fig. 1. Opposing effect of NF- κ B during PCD after cytokine withdrawal versus TNF- α administration. A, cytokine withdrawal induced apoptosis of FL5.12 cells. For both assays, a minimum of 200 cells per field and at least two independent fields were counted. ■, viability; ●, apoptotic index. B, cytokine withdrawal-induced κ B-enhancer-dependent transcription was abrogated by the presence of I κ B- α Δ N. Parent cells (□ and ■) and stably expressing clones of I κ B- α Δ N (○) were transfected transiently with a κ B enhancer that drives a luciferase cDNA reporter (2 κ Btk/Luc), or a κ B enhancer bearing a mutation in the DNA-binding site/luciferase reporter (Mut-2 κ Btk/Luc). Results represent the average of four independent transfections in which the SD was <15%. At the indicated times, the levels of κ B-directed luciferase activity were determined. C, I κ B- α Δ N protected FL5.12 cells from cytokine withdrawal-induced apoptosis. FL5.12 cells [wild-type (■) and stably expressing I κ B- α Δ N (○) lines] were deprived of cytokine for the indicated times, and trypan blue staining was assayed. Results represent a minimum of four independent experiments. D, stable expression of I κ B- α Δ N increases TNF- α -induced apoptosis in FL5.12 cells. TNF- α alone did not cause PCD in FL5.12 cells (data not shown) until supplemented with a nonlethal dose of the protein synthesis inhibitor cycloheximide: at $t = 0$, cells were treated with 100 ng/ml rTNF- α (R&D Systems) and 0.25 μ g/ml cycloheximide. Results represent a minimum of three independent experiments. Bars, SE.



For dose-dependence analysis, 5×10^6 cells were transfected with 3 μ g of CMV EGFP (Clontech) and pSFFV-I κ B- α Δ N, RSV-RelA, or RSV-cRel in varying amounts as indicated. The total DNA transfected was normalized with empty vector. Thirty-six to 48 h after transfection, cells were resuspended in medium without IL-3. The ratio of GFP⁺ cells to the total number of cells counted was determined at $t = 0$ and $t = 1$ day after cytokine withdrawal. The ratio at $t = 0$ was normalized to 100%, and the corresponding value was determined at $t = 1$ day for each individual experiment. A minimum of 200 cells were counted in at least three independent experiments.

Bone marrow B cells were purified from femurs by an established macrophage depletion and complement lysis protocol (13). Characterization of the I κ B- α Δ N transgenic mice will be described elsewhere.⁴

Protein Collection and Western Analysis. At indicated times, cells were washed once with ice-cold PBS (all centrifugations were $800 \times g$ for 5 min), and cell pellets stored at -80°C until further use. Protein isolation was carried out at 4°C . Pellets were thawed on ice in 1.0 ml of $1 \times$ Wu buffer [Ref. 14; $2 \times$ Wu buffer stock: 20 mM HEPES (pH 7.40), 3 mM MgCl_2 , 0.2 mM EGTA, 10% glycerol, 100 mM β -glycerophosphate, 1 mM DTT, 2 μ M pepstatin A, 1.0 mM phenylmethylsulfonyl fluoride], pipetted vigorously, allowed to stand for 10 min, and pipetted again. The samples were centrifuged at $11,000 \times g$ for 30 min. Supernatants were collected as cytoplasmic fractions. Nuclei were washed in 1.0 ml of $1 \times$ Wu buffer, centrifuged for 15 min, resuspended by shearing through an 18-gauge needle in $1 \times$ Wu buffer supplemented with 450 mM KCl, and centrifuged for 30 min. These supernatants and the cytoplasmic fractions were dialyzed overnight with buffer D [20 mM HEPES (pH 7.90), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 50 mM β -glycerophosphate, 1.0 μ M pepstatin A]. Dialysates were centrifuged for 30 min, and Bradford analysis (Bio-Rad) was conducted to determine the protein concentration. Equal concentrations of cytoplasmic (100–150 μ g) or nuclear extracts (50–75 μ g) were fractionated by SDS-PAGE prior to electroblot transfer to polyvinylidene fluoride Immobilon membrane (Millipore). Membranes were immunoblotted with the following antisera: I κ B- α (sc-203), cRel (sc-71), RelA (sc-109). Cytoplasmic and nuclear lysates, as indicated, were fractionated by SDS-PAGE prior to electroblot transfer to polyvinylidene fluoride membrane. Individual membranes were processed by immunoblot with the indicated antisera, and immunoglobulin/protein complexes were visualized by chemiluminescent detection (Renaissance; NEN Life Science Products).

⁴ F. E. Yull, C.-L. Chen, U. S. Sohler, D. J. Hicks, H. Li, J. O. Price, and L. D. Kerr. Inhibition of Rel/NF- κ B activity results in aberrant development and function of the B cell compartment *in vivo*, manuscript in preparation.

Results and Discussion

As established previously, FL5.12 cells undergo PCD after withdrawal of IL-3 from the medium (Fig. 1A; Refs. 10, 11). To examine whether nuclear κ B-enhancer activity is regulated during the process of cytokine withdrawal-induced apoptosis, FL5.12 cells were transfected with reporter constructs bearing two NF- κ B-inducible enhancer elements from the IL-2 receptor- α chain gene that drives expression of the *Photinus* luciferase cDNA. A transient rise in nuclear NF- κ B enhancer activity resulted in a 7-fold increase in κ B-activity, which peaked 6–8 h post cytokine withdrawal and gradually diminished to baseline over 48 h (Fig. 1B). A reporter construct bearing the HIV-1 3' long terminal repeat, another κ B-responsive promoter that drives the luciferase cDNA, gave qualitatively similar results (data not shown). No transcriptional activity was detected using the mutant κ Btk-enhancer/reporter, a construct with two DNA-binding mutants of the κ B-enhancer elements. Transfected control reporter plasmids driven by the activator protein-1 enhancer construct (2XTRE/Luc) or the SP-1 enhancer construct (SP-1/Luc) were also unaffected (data not shown).

To determine whether NF- κ B factors are necessary for cytokine withdrawal-induced apoptosis, a transdominant inhibitor of the NF- κ B complex, I κ B- α Δ N (9), was stably transfected into the FL5.12 cell line, and two separate expressing clones were analyzed for their

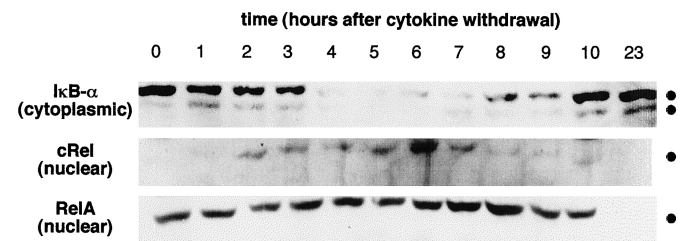


Fig. 2. Protein expression after withdrawal of cytokine from FL5.12 cells. Cytokine withdrawal induced degradation of endogenous I κ B- α and concomitant nuclear import of cRel. Levels of RelA remained constant during this same temporal window. Shown are representative films from a minimum of two independent Western analyses from at least two separate time point collections.

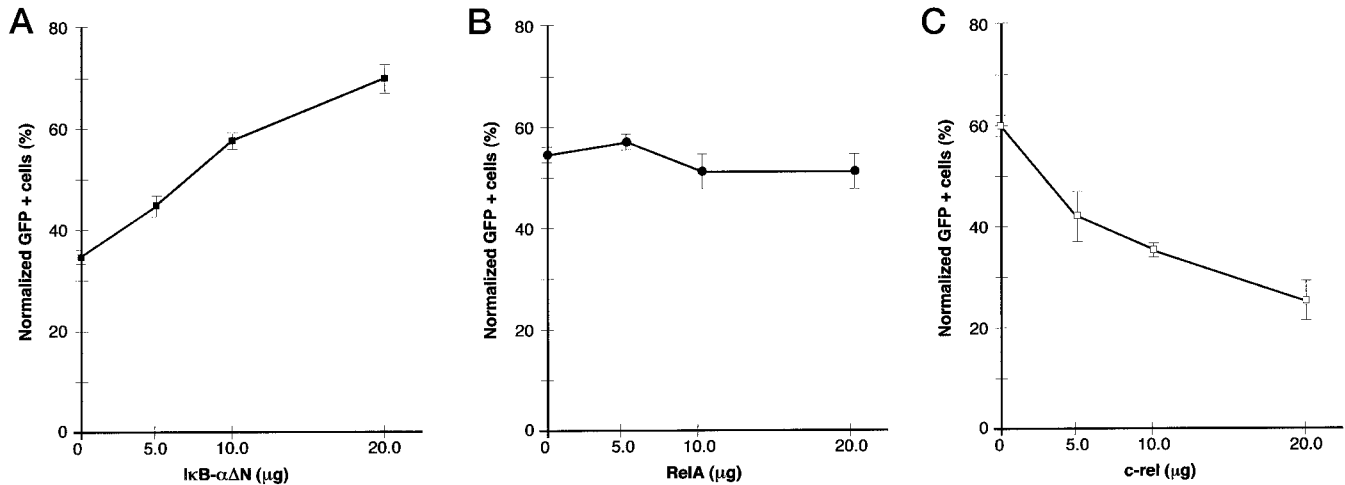


Fig. 3. Dose-dependent antagonistic effect of I κ B- α Δ N and *c-rel*. Please see "Materials and Methods" for experimental details. The *X* axis shows increasing amounts of expression vector as indicated; the *Y* axis represents the viable cells as determined by percentage of normalized GFP⁺ cells 1 day after transfection. A, increasing dose of I κ B- α Δ N caused increased survival of FL5.12 cells. B, RelA had no dose-dependent effect on the survival of FL5.12 cells. C, increased level of *c-rel* expression vector caused decreased survival in FL5.12 cells. Results represent sets of six (I κ B- α Δ N) or three (RelA and cRel) independent experiments; bars, SE.

response to cytokine deprivation. Fig. 1B compares the expression of the 2 κ Btk/Luc reporter in wild-type *versus* stably expressing I κ B- α Δ N cells and confirms that I κ B- α Δ N inhibits the transactivating capabilities of NF- κ B. A comparison of cell survival rates after cytokine withdrawal between wild-type and stably transfected I κ B- α Δ N FL5.12 cells showed that inhibition of NF- κ B-induced activity significantly decreased the onset of apoptosis (Fig. 1C). These results demonstrate that cytokine withdrawal induces NF- κ B activity, which may regulate downstream target genes critical in modulating a proapoptotic pathway. Recent reports have implicated NF- κ B as an antiapoptotic factor in the TNF- α -induced PCD pathway (2–4). Consistent with previous observations, inhibition of NF- κ B activity in FL5.12 post TNF- α treatment resulted in cells that died twice as fast as parental cells (Refs. 2–4; Fig. 1D). Taken together, these data suggest that there are multiple pathways leading to death within the same cell type and that transcription factor NF- κ B can either promote or attenuate PCD, depending on the pathway activated.

To identify the NF- κ B family member responsible for apoptosis after cytokine withdrawal, immunoblot analyses were performed on protein extracts prepared from FL5.12 cells at various times after cytokine withdrawal. Consistent with current models for activation of NF- κ B, the cytoplasmic protein levels of the major inhibitor molecule, I κ B- α , decreased beginning ~2 h after the removal of cytokine (Fig. 2). Reduced levels of I κ B- α are observed for ~4 h. No significant alteration was observed in the cytoplasmic protein levels of the other major NF- κ B inhibitor molecule, I κ B- β (data not shown). Coordinately with the disappearance of I κ B- α , the nuclear translocation of the *c-rel* proto-oncogene product, cRel, was detectable ~3 h after cytokine withdrawal. Detection of nuclear cRel peaked at 6 h and diminished concomitant with the resynthesis of I κ B- α (by 8 h). No significant alteration in the nuclear level of RelA protein was observed until 23 h post cytokine withdrawal. No alteration in cytoplasmic I κ B- α , nuclear cRel, or RelA proteins was detected in extracts from FL5.12 cells stably expressing I κ B- α Δ N, after cytokine withdrawal (data not shown).

To address directly the role of either cRel or RelA in cytokine withdrawal-induced apoptosis in FL5.12 cells, we modified a method used previously to quantify transfected cells undergoing apoptosis via visualization of a constant level of GFP expression vector cotransfected with increasing levels of a plasmid expressing

a factor of interest (15). Viable cells were GFP⁺. As observed with stable I κ B- α Δ N clones, increasing levels of I κ B- α Δ N expression vector resulted in a dose-dependent increase in GFP⁺ cells counted 1 day post cytokine withdrawal (Fig. 3A). Because the presence of I κ B- α Δ N blocks NF- κ B activation, these results confirm that NF- κ B acts in a proapoptotic manner in FL5.12 cells. Whereas RelA has been hypothesized to generate antiapoptotic signals (2–4), in this model of progenitor B-cell apoptosis, increasing concentrations of RelA expression vector were shown to have no significant effect on cytokine withdrawal-induced apoptosis (Fig. 3B). This finding correlates well with the observation that RelA levels did not change throughout the early stages of cytokine withdrawal-induced apoptosis and that this high endogenous level of nuclear RelA is unable to activate an exogenous κ B-enhancer/reporter (Fig. 1B). These results would argue for a minimal function of RelA in progenitor B-cell cytokine withdrawal-induced apoptosis, consistent with observations from early B-cell lineages isolated from RelA $-/-$ mice that were at the same levels as wild-type controls (16). Consistent with our hypothesis that cRel promotes apoptotic cell death in progenitor B cells, there was a dose-dependent decrease in GFP⁺, *i.e.*, viable, cells 1 day post cytokine withdrawal with increasing levels of *c-rel* expression vector (Fig. 3C). In this regard, it is interesting to note that we were unable to generate stable clones that expressed cRel because these cells underwent PCD even in the presence of cytokine.⁵ In summary, (a) cRel translocates to the nucleus in the early phase after cytokine withdrawal (Fig. 2); (b) sequestering of NF- κ B in the cytoplasm delays apoptosis (Fig. 1C and Fig. 3A); and (c) increasing levels of cRel decrease viability of cells after cytokine withdrawal in a dose-dependent manner (Fig. 3C). Taken together, these data demonstrate that cRel promotes death in early progenitor B cells after cytokine withdrawal.

To confirm the proapoptotic role of NF- κ B during cytokine withdrawal in early B cells in a physiological context, we generated four transgenic mouse lines that expressed the transdominant negative NF- κ B inhibitor I κ B- α Δ N in B cells.⁴ To mimic cytokine withdrawal, we purified bone marrow B cells from nontransgenic and transgenic mice, plated them in a rich B-cell medium, and monitored their viability over

⁵ U. S. Sohur and L. D. Kerr, unpublished material.

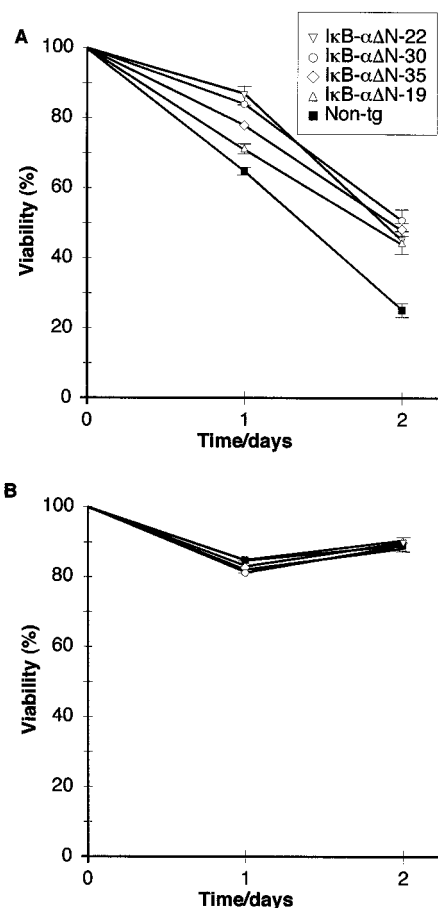


Fig. 4. Delayed death kinetics in *ex vivo* cultured transgenic I κ B- α Δ N bone marrow B cells. Death curves from control (*non-tg*; ■) and from four independent transgenic lines are shown. A, nontransgenic B cells underwent PCD with faster kinetics when cultured in rich B-cell medium (FL5.12 minus IL-3) after harvest from bone marrow. B, the differential death pattern between I κ B- α Δ N and nontransgenic B early B cells was abolished if cells were provided with an artificial bone marrow environment in the form of the S10 stromal cell layer supplemented with rIL-7 (Biosource International). Results are representative of at least three independent experiments done in triplicate, and SE values (bars) are shown where significant.

time. As would be predicted from the data obtained in FL5.12 cells, I κ B- α Δ N transgenic B cells from all four lines had a statistically significant delayed death profile ($P < 0.0001$), compared with nontransgenic bone marrow B cells (Fig. 4A). Survival of precursor B cells in the bone marrow is supported by cytokines and factors provided by the bone marrow stroma (17). We tested the effects of such factors on the delayed death rate of I κ B- α Δ N transgenic and control precursor B cells. Bone marrow B cells were cultured on an S10 stromal layer supplied with IL-7, which previously has been shown to mimic the bone marrow microenvironment (18). When transgenic and wild-type early B cells were cultured under these conditions, no difference was detected between these two groups (Fig. 4B). Thus, only in the absence of given cytokines did NF- κ B appear to promote apoptosis.

In mammals, the repertoire of the humoral immune system is selected by both positive and negative decisions regulated by a wide variety of intracellular and extracellular stimuli. As B cells mature and leave the bone marrow, cells deprived of growth factors are eliminated by PCD.

both tissue culture and cells from *in vivo* model systems, inhibition of NF- κ B activity correlated with a resistance to cytokine withdrawal-induced PCD. In addition, the data suggest that c-Rel may be the NF- κ B family member responsible for promotion of apoptosis. Our studies suggest that the critical physiological function of B-cell development is regulated, in part, by activities controlled through NF- κ B.

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