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NF-κB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type

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Abstract

Recently NF-κB has been shown to have both proapoptotic and antiapoptotic functions. In T cell hybridomas, both T cell activators and glucocorticoids induce apoptosis. Here we show that blockade of NF-κB activity, using a dominant negative IκBα, has opposite effects on these two apoptotic signals. Treatment with PMA plus ionomycin (P/I) results in the upregulation of Fas Ligand (FasL) and induction of apoptosis. Inhibition of NF-κB activity inhibits the P/I mediated induction of FasL mRNA and decreases the level of apoptosis in these cultures, thus establishing NF-κB as a proapoptotic factor in this context. Conversely, inhibition of NF-κB confers a tenfold increase in glucocorticoid mediated apoptosis, establishing that NF-κB also functions as an antiapoptotic factor. We conclude that NF-κB is a context-dependent apoptosis regulator. Our data suggests that NF-κB may function as an antiapoptotic factor in thymocytes while functioning as a proapoptotic factor in mature peripheral T cells.

Keywords: NF-κB; glucocorticoid; Fas Ligand; apoptosis; T cell; AICD; thymic selection

Abbreviations: TCR, T cell receptor; FasL, Fas Ligand; AICD, activation induced cell death; DEX, dexamethasone; mIκBα, IκBαS32/36A mutant; neo, neomycin resistance gene; P/I, PMA plus ionomycin

Introduction

In order to ensure an appropriately functioning immune system which can distinguish between self and non-self, a number of different signals have evolved to induce lymphocyte apoptosis. Endogenous glucocorticoids are thought to mediate apoptosis of thymocytes with non-functional T cell receptors (TCR) during positive selection.[1] In turn, apoptosis, initiated by activation of the TCR, plays an important role in negative selection of thymocytes as well as deletion of peripheral T cells.[2] Understanding the molecular basis for T cell apoptosis has been greatly aided by the development of cell culture systems, such as T cell hybridomas, amenable to biochemical and genetic manipulations. Ashwell and colleagues were the first to demonstrate activation induced cell cycle arrest followed by cell death.[3,4] This activation induced cell death (AICD) was subsequently characterized as apoptosis.[5–7] Interestingly, simultaneous exposure of T cell hybridomas to glucocorticoids and to activation results in the mutual inhibition of these two apoptotic pathways.[8,9] Indeed this observation led to the discovery that glucocorticoids are produced in the fetal thymus and function to induce thymocyte apoptosis during selection.[10,11] In addition this work led to the observation that glucocorticoids inhibit anti-CD3 mediated induction of Fas Ligand (FasL) expression.[12]

While glucocorticoid mediated thymocyte apoptosis remains largely undefined, AICD in peripheral T cells has been intensively studied. Analysis of the lpr and gld mutations, both resulting in a lymphoproliferative disorder primarily inhibiting peripheral T cell deletion,[13,14] has resulted in the association of this phenotype with the dysfunction of the Fas receptor (Fas) and the FasL respectively.[15] Analysis of AICD in several culture models including T cell hybridomas subsequently identified Fas/FasL interactions as important for the induction of apoptosis.[16–18] Fas is a member of the TNF receptor family which includes TNFR1, TNFR2, as well as receptors for lymphotoxin-β, CD27, CD30, CD40 and others.[15] A subgroup within this family which includes Fas and TNFR1 have a conserved cytoplasmic domain, referred to as the death domain, which is necessary for initiation of apoptosis. A growing number of proteins have been identified which interact with the Fas and TNFR1 death domains.[19–21] Upon engagement of the FasL, Fas associates with FADD (MORT1) which itself contains a C-terminal death domain.[22,23] The N-terminus, which contains the death effector domain (DED) interacts with pro-caspase 8 (MACH, FLICE), ultimately resulting in the activation of a caspase cascade which mediates apoptosis.[24,25] The death domain of TNFR1 associates with TRADD and with RIP.[26,27] TRADD, in turn, interacts with FADD, suggesting that the apoptotic signals initiated by FasL and TNFα ultimately converge.[28]

TRADD also induces the transcription factor NF-κB by interacting with TRAF proteins.[29] NF-κB plays an important role in the activation of responses to immune challenge, inflammation, and cellular stress.[30,31] In addition, NF-κB has recently been shown to play an important and varied

role in the regulation of apoptosis. NF-κB is comprised of a heterodimeric complex of a 50-kDal subunit (p50, NF-κB1) and a 65-kDal subunit (p65, RelA) which is complexed in the cytoplasm with a family of inhibitors termed IκBs.[30,31] The NF-κB p50 and RelA subunits, along with p52 (NF-κB2, lyt-10) and RelB, share homology with the products of the c-rel oncogene and *Drosophila* maternal effects gene, Dorsal, thus forming the Rel/NF-κB gene family.[30,31] The N-terminal region of homology, termed the Rel homology domain, mediates dimerization, DNA binding, nuclear localization, and interaction with IκB through IκB's ankyrin repeat domain. These NF-κB genes are able to homodimerize and heterodimerize to form a large number of NF-κB-like activities which have been shown to have differing affinities for various NF-κB response elements.[32] Numerous stimuli including mitogens, cytokines, reactive oxygen intermediates, LPS, and viral infection result in the activation of NF-κB. In many instances this is thought to involve the activation of the IκB kinase (IKK) complex which then phosphorylates IκB, resulting in its ubiquitination and degradation by the proteosome.[33–35] Dissociation of IκB from NF-κB allows NF-κB to translocate to the nucleus. Thus NF-κB-like activities comprise a complex web of interrelated factors which can regulate subsets of a large number of genes in response to specific stimuli.

In a number of systems, NF-κB has been demonstrated to have an antiapoptotic function. Knockout of relA results in an embryonic lethal phenotype which was found to be caused by TNFα induced apoptosis within the fetal liver.[36] Subsequently, it was demonstrated that reintroduction of relA to immortalized hepatocytes derived from these animals protected these cells from TNFα induced apoptosis.[37] NF-κB has also been shown to inhibit TNFα mediated apoptosis in primary rat and human fibroblasts as well as Jurkat T cells and the T24 human bladder carcinoma line[38] and in MCF7 breast cancer cells.[39] In addition, NF-κB has been shown to play a role in apoptosis mediated by engagement of PPARα and PPARγ in both TNFα stimulated and in unstimulated differentiated macrophages.[40] NF-κB has also been found to protect ras transformed tumor cells from apoptosis induced by chemotherapy.[41,42] Several NF-κB responsive antiapoptotic genes which may play a role in this process have subsequently been identified and include cIAP1, cIAP2, TRAF1 and TRAF2.[43] In addition IEXL-1 has been identified as an NF-κB responsive antiapoptotic gene[44] however the mechanism by which this protein blocks apoptosis is unclear. Regulation of NF-κB has also been shown to play a role in both TGFβ mediated induction of B cell apoptosis through induction of IκBα expression[45] as well as in CD40L rescue from apoptosis through decreased IκBα expression.[46] Here NF-κB functions to stimulate expression of c-myc, a factor necessary for B cell survival. Thus NF-κB plays an antiapoptotic role in a variety of systems and activates different subsets of antiapoptotic genes within these systems.

Evidence has also accumulated implicating NF-κB in the induction of apoptosis. Studies have correlated induction of apoptosis with activation of NF-κB in a wide variety of systems including avian embryonic development,[47] ceramide activated osteoblasts[48] and dopaminergic neurons derived from Parkinson disease patients.[49] With the development of inhibitors of NF-κB function, more definitive evidence has since been generated. Alphavirus induction of apoptosis in a prostate carcinoma line was shown to be inhibited by inhibiting NF-κB activity using binding site decoys.[50] Interestingly, viral induction of apoptosis in a neuroblastoma line was not affected by inhibition of NF-κB, underscoring the cell specificity of NF-κB mediated regulation of apoptosis. Similarly, Denge virus induction of apoptosis in hepatocytes could be blocked using NF-κB decoys.[51] Within the CNS, pathological activation of glutamate receptors and the subsequent generation of oxygen radicals may be a pathway leading to an apoptosis-like process of neurodegeneration common to several disease states.[52] Evidence for NF-κB involvement came from the observation that aspirin both acts as a neuroprotective agent and functions to inhibit glutamate receptor mediated activation of NF-κB.[53] More recent studies then confirmed the role of NF-κB by showing that a cell permeable NF-κB blocking peptide inhibited quinolinic acid-induced striatal cell death.[54] Thus the role of NF-κB as a regulator of apoptosis is complex and tissue specific.

NF-κB activity has been detected in both thymocytes and mature T cells.[30,31] Studies of specific NF-κB subunits expressed during thymocyte positive selection have identified a strong upregulation of c-Rel mRNA and an increase in the constitutive activity of several forms of NF-κB.[55] In mature peripheral T cells representing murine Th1 and Th2 populations, NF-κB activity was found to be much decreased in the Th2 population as compared to Th1 and Th0 populations. Thus NF-κB changes both in subunit composition and activity level during T cell development. Nuclear translocation of NF-κB in mature peripheral T cells requires both engagement of the TCR as well as a co-stimulatory signal. In turn, T cell activation requires the *de novo* transcription of IL-2, which is regulated by NF-κB, NFAT, and AP-1 activities.[56,57] Knockout of specific NF-κB genes results in reduced peripheral T cell activation and proliferation, but little change in thymic selection.[58] Recently, through the study of NF-κB activation via IκBa phosphorylation, serines at positions 32 and 36 were identified as critical targets for IKK mediated activation.[59,60] An IκBα mutant was then developed replacing serines 32 and 36 with alanines. This mutant binds to NF-κB but cannot release it in response to stimulation and thus effectively inhibits all inducible forms of NF-κB activity. In contrast to the knockout experiments, direction of the expression of this mutant IκBα (mIκBα) to thymocytes of transgenic mice, results in a significant loss of T cells which survive thymic selection as well as a relative increase in the ratio of CD4 to CD8 single positive T cells,[61] suggesting that NF-κB plays an important role in the selection process and that different forms of NF-κB may be able to substitute for each other. Hence NF-κB plays a role both in the processes of thymic selection as well as peripheral T cell activation.

Recently, it has been shown that glucocorticoids can inhibit NF-κB activity through direct association of GR with NF-κB subunits,[62–64] through induction of IκBα expression,[65,66] and through competition for the co-activator

protein, CBP/p300.[67] This interaction between two transcription factors, both of which function to regulate apoptosis, has led us to hypothesize that NF-κB may play an important role in modulating glucocorticoid mediated apoptosis as well as AICD. Here we demonstrate that inhibition of NF-κB leads to loss of the induction of FasL expression and reduced apoptosis while simultaneously increasing the sensitivity of these cells to DEX mediated apoptosis. Thus NF-κB has both proapoptotic and antiapoptotic functions within a single cell type.

Results

Inhibition of NF-κB in T cell hybridomas

As mentioned above, IκBα phosphorylation is essential for NF-κB activation. An IκBα mutant in which serines in positions 32 and 36 have been substituted for alanines (mIκBα) has been developed by several laboratories to function as a strong dominant negative inhibitor of NF-κB.[59,60] We introduced this IκBα mutant into the T cell hybridoma lines, D011.10 and 2B4.11 by electroporation of either pCMV4-IκBαS32/36A or pCMV4-FLAG-IκBαS32/36A (gifts of D. Ballard) in the presence of the neomycin resistance gene and established a number of independent stable mIκBα expressing lines as described in Materials and Methods. The neomycin resistance gene (neo) was also transfected alone to create several control lines. Presence of the FLAG epitope has no effect on mIκBα function (D. Ballard, personal communication) but allows for the measurement of transgene detection apart from endogenous IκBα expression. Figure 1 shows a representative Western blot analyzing the expression of the FLAG epitope tagged transgene in several 2B4.11 lines. The transgene migrates just below the 41.8 kDal molecular weight marker as expected (Figure 1). Lines 2P, 4P, and 5P are representative of high FLAG epitope expression while lines 3P and 5M are representative of intermediate FLAG epitope expression (Figure 1). Lines 3M and 4M have very low, but detectable, transgene expression while parental 2B4.11 cells did not contain the FLAG epitope (Figure 1). Neo expressors generated a pattern identical to parental lines (data not shown).

We then wanted to characterize NF-κB expression in these cells. 2B4.11 cultures were treated with PMA and ionomycin (P/I) and nuclear extracts prepared as described in Materials and Methods. EMSA analysis was then performed using an NF-κB site, found on the FasL promoter, as a probe. As shown in Figure 2A, treatment of parental 2B4.11 cells with P/I resulted in the up regulation of a protein/DNA complex which was present in minor amounts in non-activated cells. This complex was effectively blocked by a 20-fold excess of unlabeled DNA corresponding to an NF-κB site derived from the murine H2-K^b MHC class I promoter (κB). In contrast, a 20-fold excess of a double point mutant of the H2-K^b NF-κB site (mκB) was unable to effectively block the formation of the complex. The FasL NF-κB site (FL) also competed against self, as expected. To further confirm the identity of this DNA binding protein, we performed a supershift experiment. As shown in Figure 2B, pretreatment with antisera specific for relA(p65) partially supershifted the complex while an antisera specific for p50 blocked DNA binding, suggesting that the complex contains of heterodimers of relA and p50. While the presence of other NF-κB subunits in this complex has yet to be determined, it is clear from the data presented that this complex corresponds to NF-κB. We then tested the ability of the mIκBα transgene to block the nuclear translocation of NF-κB. As shown in Figure 2C, NF-κB DNA binding is induced upon treatment of a Neo P/I expressing line. Induction of NF-κB binding in the 2B4.11 4P line is greatly decreased however by overexpression of the transgene (compare Figure 1 and Figure 2C). Interestingly, a low mIκBα expressing line, 3M, was able to translocate NF-κB after P/I treatment indicating that the decrease in NF-κB translocation requires expression of the transgene and is not just caused by the selection process.

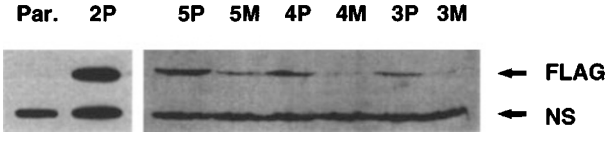

Figure 1 2B4.11 T cell lines expressing various levels of FLAG-tagged mIκB. Cytosolic extracts of the parental 2B4.11 line and various 2B4.11 lines expressing a FLAG-tagged mIκBα transgene were examined by FLAG antibody Western and visualized by ECL. NS: non-specific binding. Lines 2P, 3P, 4P, and 5P were isolated from one transfection experiment. Similarly, lines 3M, 4M, and 5M were isolated from a separate transfection experiment

FasL expression is dependent on NF-κB translocation

The study of Yang and colleagues[12] suggested to us that glucocorticoid mediated inhibition of FasL may involve inhibition of NF-κB. In addition, cloning of FasL upstream sequence uncovered a putative NF-κB site.[68] To determine the effect of the inhibition of NF-κB translocation on FasL expression, we first examined D011.10 cultures transfected with pCMV4-IκBαS32/36A using RT–PCR. Since this construct lacks an epitope tag, we were unable to follow transgene expression by Western blot. Figure 3A (lanes 1–7) displays a representative experiment. Cells were untreated or treated with P/I for 2 h and RNA prepared by the acidified phenol method.[69] RT–PCR was then performed using primers specific for FasL mRNA (Figure 3A, top) and for β-actin mRNA (Figure 3A, bottom) as described in Materials and Methods. As shown in Figure 3A, lane 2, untreated D011.10 cultures did not express detectable FasL transcripts. After P/I treatment however, a robust band was detected corresponding exactly to the size expected for FasL (compare Figure 3A, lane 3 with Figure 3B, lane 8). We obtained several pCMV4-IκBαS32/36A transfected D011.10 lines in which FasL expression could no longer be induced by P/I treatment (compare Figure 3A, lane 3 with lanes 6 and 7). In comparison, expression of the neomycin resistance gene had no effect on FasL expression (compare lane 3 with lanes 4 and 5). In a separate experiment (Figure 3A, lanes 8–12), another pCMV4-IκBαS32/36A transfected D011.10 line, IκB2, was found to express low levels of FasL in response to P/I

treatment as compared to parental and Neo expressors (compare Figure 3A lanes 9–11). Once again, the IκB4 line was unable to express FasL mRNA (Figure 3A, lane 12). All cellular derived cDNAs amplified roughly equivalent amounts of the 300 bp β-actin fragment indicating that lack of amplified FasL is not due to RNA degradation (Figure 3A and B, bottom panels). Interestingly, several weeks later, several of these pCMV4-IκBαS32/36A transfected D011.10 lines regained their ability to generate FasL mRNA in response to P/I treatment (data not shown), suggesting the possibility that the transgene was no longer expressing properly. This prompted us to generate new lines containing the mutant IκBα tagged with the FLAG epitope as described above and as shown in Figure 1. As shown in Figure 3B, expression of the FLAG tagged transgene in 2B4.11 T cell hybridomas (line 4P) resulted once again in the loss of FasL mRNA expression while expression of the neomycin resistance gene had no effect (Figure 3B, compare lane 3 with lanes 5 and 7). Because of the genetic instability of these T cell hybridoma cultures, these lines were screened regularly by Western blot (Figure 1). Transgene expression was often lost within 1–3 months of culture. In order to control for potential changes in the expression level of the transgene, each treatment group was divided into equal aliquots and examined at several levels. Expression of FasL mRNA abundance was determined by RT–PCR as described above. Loss of expression of the mIκBα transgene invariably resulted in reappearance of the FasL mRNA upon P/I treatment further suggesting that FasL expression is specifically inhibited by the functional inhibition of NF-κB (data not shown).

The second matched aliquot of cells was analyzed for functional FasL protein. To this end, our variously treated 2B4.11 cultures (effector cells) were mixed with ^{51}Cr loaded L1210-Fas cells, which express high levels of Fas (target cells). Expression of functional FasL protein on the effector cell surface will cause target cell lysis and release of ^{51}Cr into the culture medium. All untreated 2B4.11 lines induced target cell lysis indistinguishable from the spontaneous lysis rates (Figure 3C). Parental and neo over-expressors, when treated with P/I and co-cultured with the target cells, induced significantly higher rates of lysis (averages of 56 and 70% respectively in the illustrated experiment). Lines expressing high levels of mIκBα however, upon treatment with P/I, showed no significant increase in target cell lysis, indicating a lack of expression of functional FasL (Figure 3C). Consistent with the PCR data above, low mIκBα expressing lines showed intermediate lysis of targets upon P/I treatment, while loss of transgene expression correlated with significant levels of target cell lysis upon P/I treatment (data not shown). We conclude from these experiments that these cells express functional FasL upon activation and that this expression requires the activation of NF-κB.

While this work was in progress, a report was published demonstrating that cell damaging agents such as teniposide and etoposide induce expression of FasL in Jurkat T cell cultures through induction of NF-κB.[70] They cloned the FasL promoter and identified an NF-κB site approximately 1100 bp upstream from the transcription start site. Mutation of this site blocked the ability of DNA damaging agents from inducing the promoter. This work demonstrated that

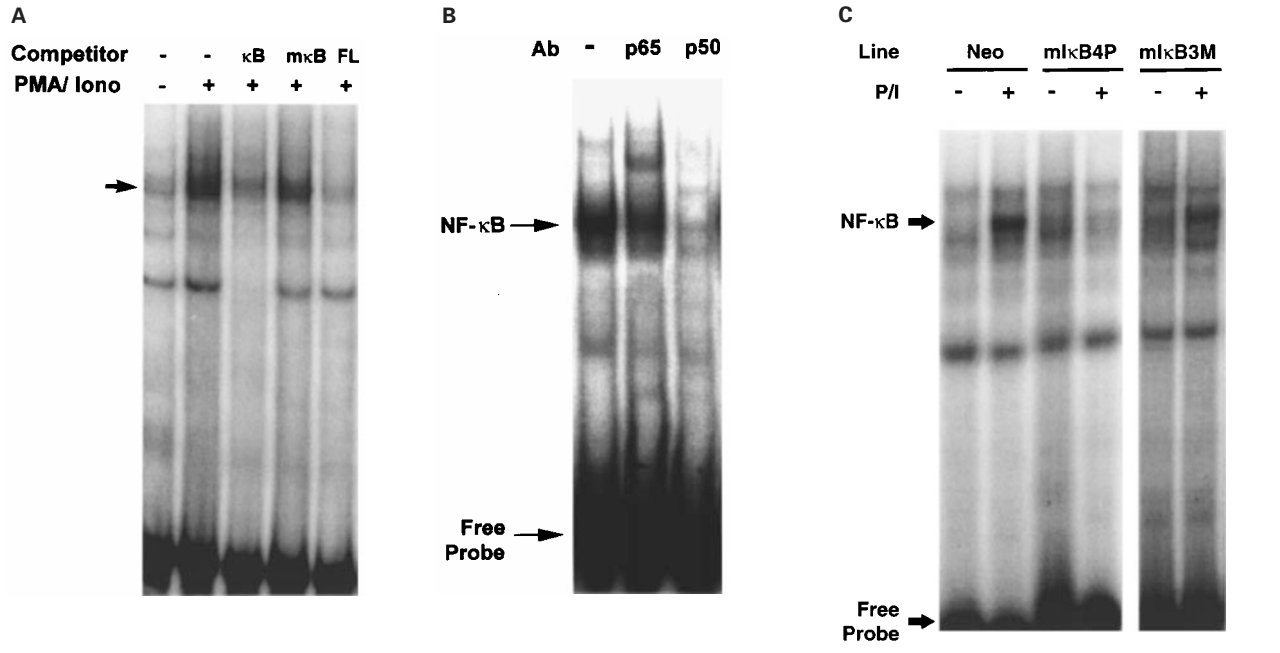

Figure 2 Analysis of NF-κB DNA binding in 2B4.11 parental and transfected lines. (**A**) Nuclear extracts from control and PMA plus ionomycin (PMA/iono) activated 2B4.11 parental cultures were incubated with a probe encoding the FasL promoter NF-κB site in the presence of various unlabeled DNA competitors. Results were examined by EMSA and visualized by Phosphor Imager. κB: NF-κB site from the H2-K^{b}MHC class I promoter, κm: mutated κB sequence, FL: FasL NF-κB site. The arrow marks the location of the NF-κB complex. (**B**) Nuclear extract from a PMA/ionomycin activated 2B4.11 culture was preincubated with antibodies specific for NF-κB relA (p65) or p50 subunits and analyzed as in (**A**). (**C**) A 2B4.11 neo expressor, line 4P, and line 3M were either untreated or activated with PMA/ionomycin (P/I) and nuclear extracts analyzed as in (**A**)

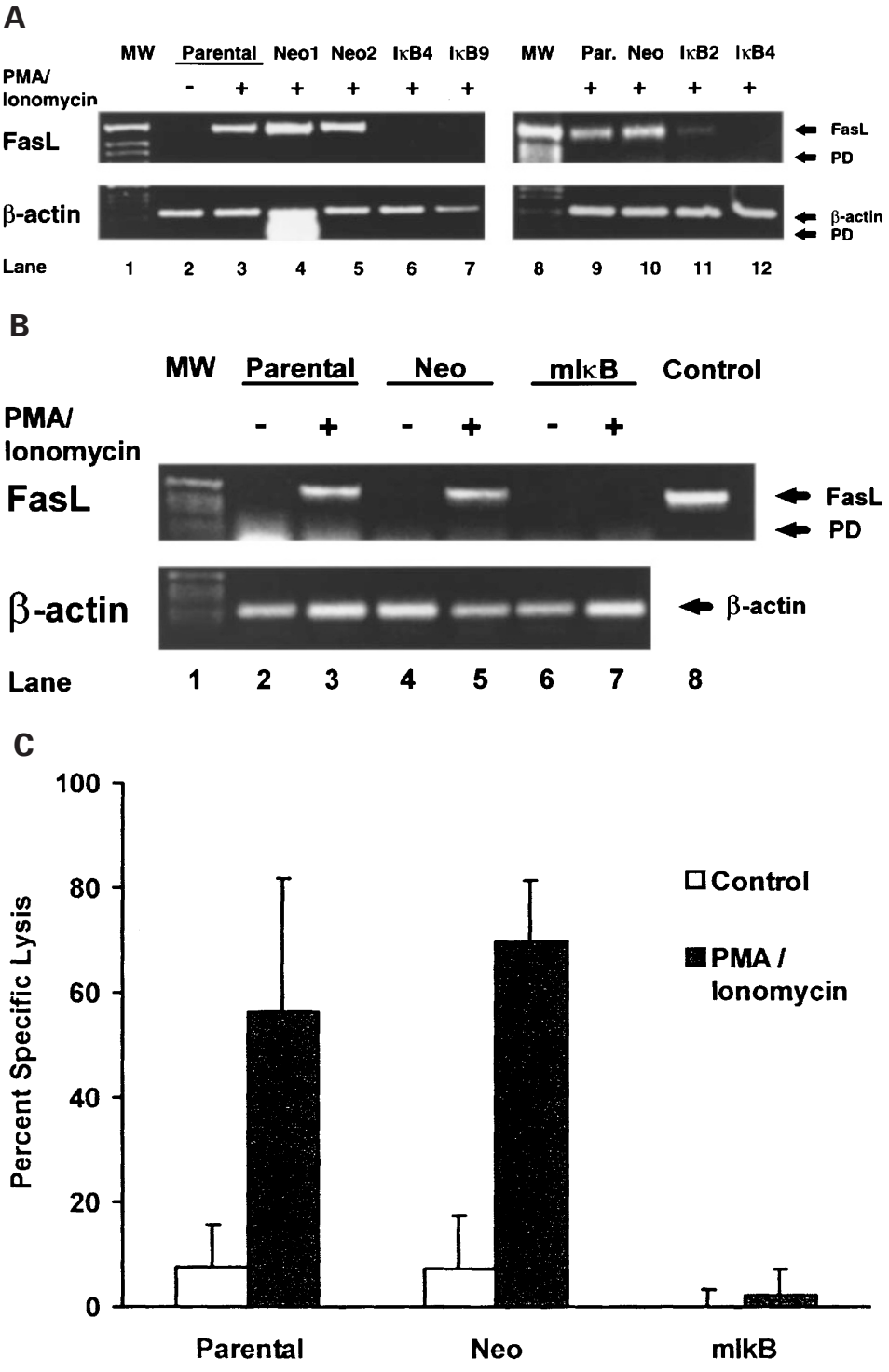

Figure 3 Analysis of FasL expression. (**A**) D011.10 parental, pCI-neo transfected lines (Neo1, Neo2), and pCMV4-IκBαS32/36A transfected lines IκB2, IκB4, and IκB9 were untreated or treated for 2 h with PMA/ionomycin. RNA was prepared and analyzed by RT–PCR for FasL (top panel) and β-actin (bottom panel) as described in Materials and Methods. MW: 1 kb ladder (Gibco). PD: primer dimer. (**B**) 2B4.11 parental, Neo1 (Neo), and 4P (mIκB) lines were treated and mRNA prepared and analyzed as in (**A**) Control: RT–PCR from a FasL cDNA plasmid, PD: Primer dimers. (**C**) PMA/Ionomycin treated and untreated 2B4.11 parental, Neo-1, and 4P (mIκB) lines were co-cultured with L1210 Fas expressing target cells pre-loaded with ^{51}Cr. Specific lysis was calculated as per methods and materials. Open bars: Untreated control, Filled bars: PMA/ionomycin treated. Data represents three independent determinations. Error bars denote standard deviation

NF-κB acts directly to induce transcription of FasL when activated by DNA damaging agents. Our work confirms this observation and demonstrates that T cell activator mediated induction of FasL is also dependent on the nuclear translocation of NF-κB.

Inhibition of NF-κB in T cell hybridomas has both proapoptotic and antiapoptotic effects depending on cellular context

Engagement of Fas has been shown to play a major role in the induction of AICD.[16–18] To determine the effect of the inhibition of NF-κB activation and FasL expression on AICD, 2B4.11 cultures were treated with either P/I or with anti-CD3 for 24 h and viability determined by trypan blue exclusion. As shown in Figure 4A, we observed 87% (±1%) killing of the parental 2B4.11 cultures after 24 h treatment with P/I as compared to matched untreated cultures. In comparison, the 2B4.11 4P line showed 49% (±3%) killing upon similar treatment. Thus the mIκBα transgene seems to function to inhibit cell death, suggesting that NF-κB activity is necessary for efficient killing to occur. P/I treatment is a powerful T cell activator which functions downstream from the TCR. In order to determine the effect of cell death mediated solely by activation of the TCR, 2B4.11 cultures were treated with anti-CD3 as described in Materials and Methods. As shown in Figure 4B, treatment of parental cultures resulted in a 32% (±4%) loss in viability after 24 h while similarly treated Neo expressors experiences a 29% (±6%) loss in viability. In comparison, the 2B4.11 4P line experienced a 13% (±5%) increase in viability. These data suggest that loss of viability, when mediated specifically through activation of the TCR, is completely blocked by overexpression of the mIκBα transgene.

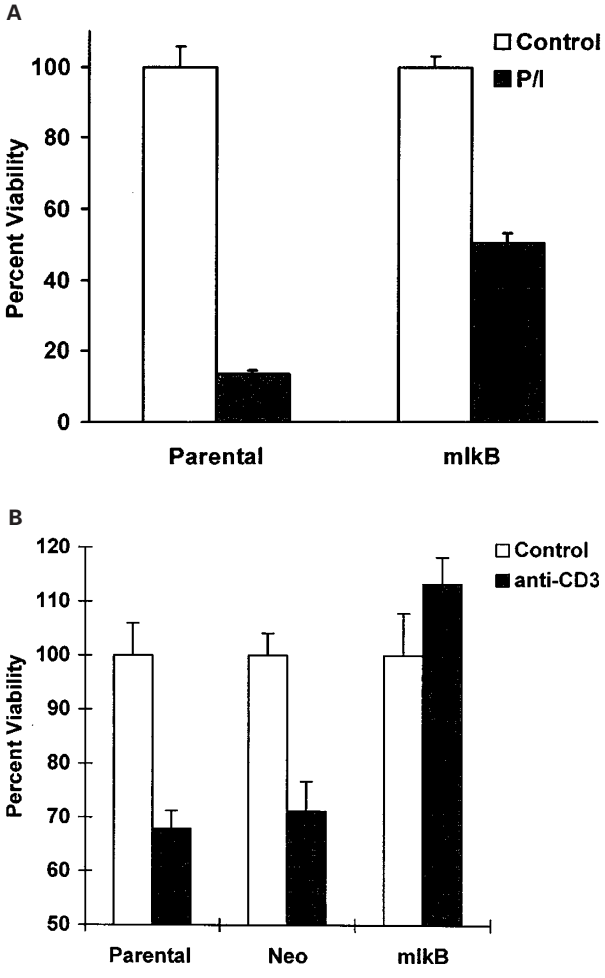

Figure 4 Effect of NF-κB inhibition on cell viability after activation. (**A**) 2B4.11 parental and 4P (mIκB) lines were untreated or treated for 24 h with PMA/ionomycin. Cell viability was assayed by trypan blue exclusion. Data is representative of three independent experiments. Error bars denote standard deviation. (**B**) 2B4.11 parental, Neo-1 (Neo), or 4P (mIκB) lines were treated with anti-CD3 for 24 h as described in Materials and Methods and cell viability measured by trypan blue exclusion. Data is representative of four independent experiments. Error bars denote standard deviation

Our viability assay cannot distinguish between increased cell death *versus* decreased proliferation. In order to determine if this increase in cell survival was due to a partial or complete inhibition of apoptosis, cells were treated with either PMA, ionomycin, or P/I for 24 h as above and then assayed for cell death visually by the ethidium bromide/acridine orange technique as described in Methods and Materials.[71] As shown in Figure 5A, neither PMA or ionomycin alone induced appreciable levels of apoptosis in parental 2B4.11 cells. Treatment of parental 2B4.11 cells with P/I however resulted in approximately 25% of the population clearly showing an apoptotic morphology while in the 2B4.11 4P line, apoptosis was indistinguishable from background. These data suggest that inhibition of NF-κB has a profound effect on the ability of T cell hybridomas to undergo apoptosis in response to direct activation of the TCR or to activation of downstream TCR signaling components.

If expression of NF-κB plays an important role in induction of apoptosis mediated by P/I treatment then we would expect a rough correlation between expression of the mIκBα transgene and protection from induction of P/I mediated apoptosis. To test this hypothesis we measured apoptosis in a variety of D011.10 and 2B4.11 lines expressing variable amounts of the mIκBα transgene. As shown in Figure 5B (left) we measured the induction of apoptosis in the D011.10 IκB2 line as compared to a Neo expressor. Since this line was created with pCMV4-IκBαS32/36A, we were unable to directly measure the expression of the transgene. Nevertheless, as shown in Figure 3A, lane 11, the IκB2 expresses significantly less FasL mRNA in response to P/I stimulation than the parental or Neo expressing cells. We found that this loss in FasL expression correlated with a partial protection from P/I induced apoptosis as compared to the Neo expressor. We then measured the induction of apoptosis in a variety of 2B4.11 lines expressing varying amounts of the FLAG epitope tagged mIκBα transgene as shown in Figure 1. Line 3P is a moderate expressor of the mIκBα transgene and as shown in Figure 5, is partially protected from P/I mediated apoptosis. In comparison, lines 3M and 4M have barely detectable levels of transgene expression and are

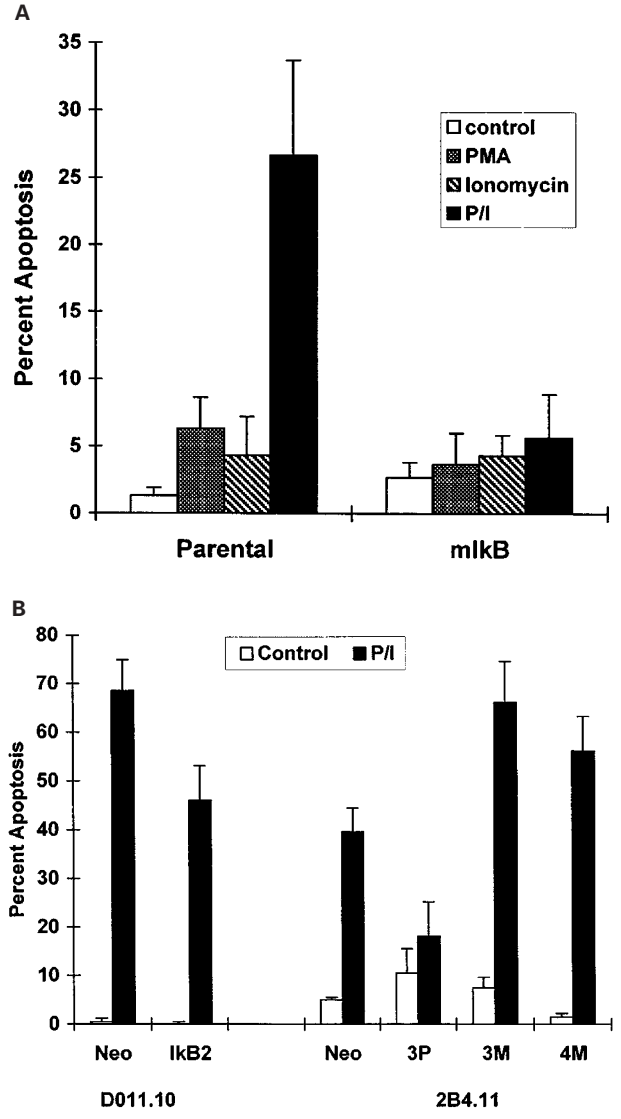

Figure 5 Effect of NF-κB inhibition on apoptosis after activation. Apoptosis was morphologically determined using the ethidium bromide/acridine orange technique.[71] Per cent apoptosis was calculated as (apoptotic population counted/total population counted) as described in Methods and Materials. Error bars denote standard deviation. (**A**) 2B4.11 parental and 4P lines were either left untreated or treated 24 h with PMA alone, ionomycin alone, or PMA plus ionomycin (P/I) as described in Materials and Methods. Data represents three independent experiments. Error bars denote standard deviation. (**B**) D011.10 lines (left) or 2B4.11 lines (right), transfected with either pCI-Neo (Neo), pCI-Neo plus pCMV4-IκBαS32/36A (IκB2), or pCI-Neo plus pCMV4-FLAG-IκBαS32/36A (3P, 3M, and 4M) were treated 24 h with P/I and apoptosis determined as in (**A**)

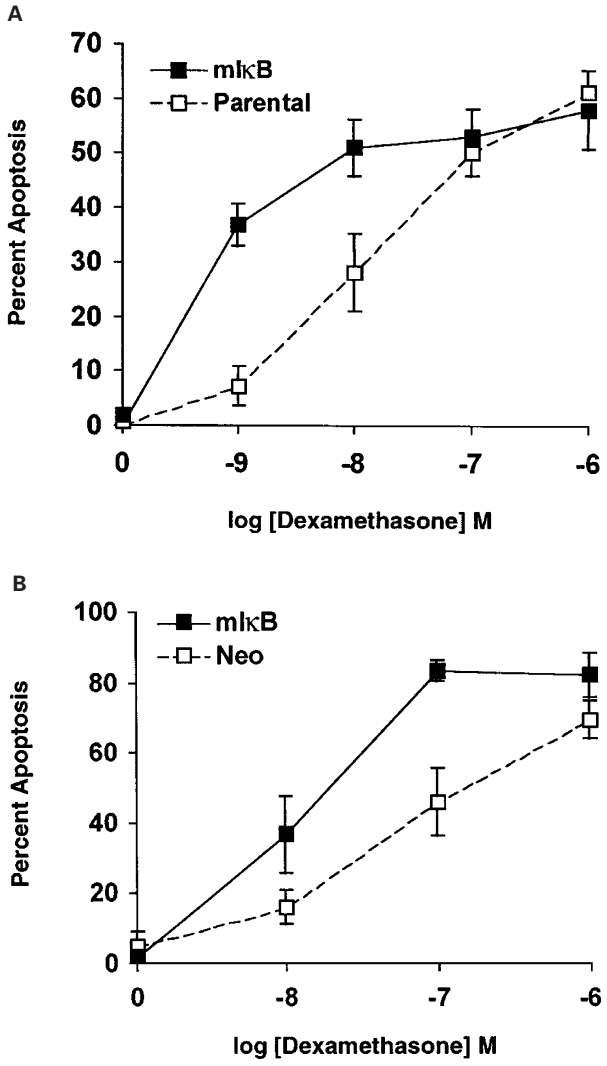

Figure 6 Effect of NF-κB inhibition on dexamethasone induced apoptosis. Apoptosis was determined as described in Figure 5. Data represents three independent determinations. Error bars denote standard deviation. (**A**) D011.10 parental and IκB9 (mIκB) lines were treated 24 h with varying concentrations of dexamethasone as shown. Filled square/solid line: IκB9, Open square/dashed line: parental D011.10 line. (**B**) 2B4.11 Neo-1 (Neo) and 4P (mIκB) lines were treated for 24 with varying concentrations of dexamethasone as shown. Filled square/solid line: 2B4.11 4P line (mIκB), Open square/dashed line: 2B4.11 Neo-1 line (Neo)

completely unprotected from the effects of P/I treatment. These data suggest that protection from apoptosis correlates directly with varying levels of FasL expression and inversely with expression of the mIκBα transgene.

Given our earlier work demonstrating the inhibition of NF-κB by glucocorticoids[63,65] as well as our current data linking NF-κB to FasL expression, we hypothesized that blockade of NF-κB might result in an increased sensitivity to glucocorticoid mediated apoptosis. In order to test this possibility, we incubated either the parental D011.10 and the IκB9 lines (Figure 6A) or the 2B4.11 Neo expressors and the 2B4.11 4P line (Figure 6B) with varying concentrations of dexamethasone (DEX) for 24 h. Apoptosis was again measured by the acridine orange/ethidium bromide technique. As expected, increasing concentrations of DEX resulted in increasing levels of apoptosis both for parental D011.10 and for 2B4.11 Neo expressing cultures in a similar fashion. Expression of the mIκB transgene in the D011.10 IκB9 line resulted in approximately a tenfold increase in sensitivity to DEX mediated apoptosis (Figure

6A). A similar increase in sensitivity was observed in our 2B4.11 4P expressing line (Figure 6B). Thus, while expression of the mIκBα transgene protects T cell hybridomas from P/I mediated apoptosis, in the context of glucocorticoid treatment, inhibition of NF-κB activity results in a significant increase in apoptosis in two different T cell lines.

NF-κB is not required for the mutual inhibition mediated by P/I and glucocorticoids

As demonstrated in earlier studies, exposure to T cell activators in the presence of glucocorticoids induces a mutual inhibition of the two apoptotic signals.[8,9] We next wanted to determine if inhibition of NF-κB had any effect on this phenomena. Parental 2B4.11, Neo-3, 3P, and 5P lines were incubated with varying amounts of DEX for 24 h in the absence or presence of P/I. Apoptosis was measured by the acridine orange/ethidium bromide technique. At the maximal dose of glucocorticoid, we counted approximately 60 to 70% of the cells as apoptotic (Figure 7). P/I treatment alone in this experiment produced 27% (±5.7%) apoptosis in our neo-3 line and 5% (±1.4%) in our 3P line (data not shown). While either DEX or P/I treatment alone induced apoptosis, when combined, apoptosis levels were indistinguishable from background (Figure 7), consistent with the observations of others.[8,9] Inhibition of NF-κB activity had a small effect, if any, on this result. Thus while inhibition of NF-κB has opposite effects on these two apoptosis pathways which are mutually inhibitory, surprisingly, NF-κB does not play a role in the mutual inhibition that occurs between these two pathways.

Discussion

A central paradox in thymic selection is that one signal can mediate survival or the induction of apoptosis depending on the strength of the signal and the differentiation state of the T cell. Our interest in this problem arose through several linked observations. In our studies of NF-κB, we discovered an interaction between the glucocorticoid receptor and NF-κB resulting in the mutual inhibition of these two transcription factors which functioned at several levels including physical interaction and induction of IκBα.[63,65] Others have more recently shown that NF-κB and GR also compete for the co-activator p300/CBP.[67] During this time, several reports demonstrated that glucocorticoids were produced in the thymic epithelium and that blockade of either glucocorticoid production or blockade of glucocorticoid receptor synthesis resulted in increased thymocyte survival during positive selection.[10,11] A previous report from the same group had demonstrated that T cell activation could block glucocorticoid mediated apoptosis.[8] Interestingly, glucocorticoid treatment was found to block anti-CD3 mediated induction of FasL.[12]

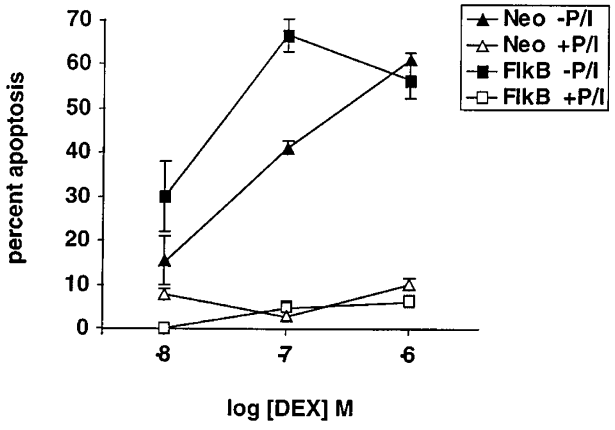

Figure 7 Effect of NF-κB inhibition on the mutual inhibition between P/I and dexamethasone induced apoptosis. The 2B4.11 Neo-3 line (triangles) and 2B4.11 3P line (squares) were treated with varying concentrations of DEX as shown in the absence (filled symbols) or presence (open symbols) of PMA/ionomycin for 24 h. Apoptosis was determined as described in Figure 5. Data represent three separate determinations and is representative of experiments with other mIκBα expressing lines. Error bars denote standard deviation

Given the recent observations that NF-κB can play a role as either antiapoptotic or proapoptotic regulator, our hypothesis, derived from these observations, was that glucocorticoid interactions with NF-κB might play a role in thymic selection. We chose to address this hypothesis by inhibiting NF-κB activity using the dominant negative mutant IκBα. The detailed apoptosis studies of Ashwell and others made the T cell hybridomas 2B4.11 and DO11.10 attractive model systems for this study. In our initial experiments we chose to look at the effects of inhibition of NF-κB on both activation induced apoptosis (AICD) and glucocorticoid induced apoptosis. Others have shown in this hybridoma model that AICD is mediated by the upregulation of FasL and its engagement with Fas.[6,12] We found that inhibition of NF-κB results in a loss of the induction of FasL mRNA in both 2B4.11 and DO11.10 (Figure 3), as well as a decrease in AICD mediated apoptosis (Figure 4 and 5). Our method of measuring apoptosis is conservative in that at the time the counts were performed, a number of cells may have already been eliminated and thus would not be counted as apoptotic. A useful experiment would be to reintroduce FasL expression to NF-κB inhibited cells and show that protection from AICD is blocked. We have attempted to perform this experiment through transient transfection techniques using our T cell hybridomas but low transfection efficiencies have stymied this attempt. In addition, while AICD was significantly decreased by inhibition of NF-κB it was never totally eradicated (Figure 5A and data not shown). This would suggest that a Fas-independent apoptotic pathway may also be activated in these cells however further work will be necessary to define these points.

While our studies were in progress several reports were published indicating that NF-κB was an important regulator of FasL. The HTLV-1 Tax protein induces the expression of FasL and promotes apoptosis in Jurkat cells.[72] Tax is known to activate NF-κB as well as the CREB/ATF transcription factor family through different domains. Interestingly, mutants of Tax which are selectively deficient in the activation of either NF-κB or of CREB/ATF are incapable of inducing the FasL. A

combination of these mutants however restores the ability to induce FasL and mediate apoptosis. This strongly suggests that NF-κB and CREB/ATF activities are necessary for the induction of FasL. Others have linked NF-κB activity with the induction of FasL through modulation of proteosome function using the bacterial metabolite, lactacystin.[73] They found that agents which inhibited NF-κB activation also inhibited induction of FasL. More recently, a study of the DNA damaging agents, etoposide and teniposide, demonstrated that in Jurkat T cells, apoptosis was mediated through the induction of FasL and that NF-κB and AP-1 activities were essential for this induction.[70] The authors cloned the FasL promoter, identified an NF-κB site between 0.9 and 1.2 kb upstream from the transcription start site, and demonstrated that mutation of that site resulted in a loss of etoposide mediated induction. This report thus supplies a mechanistic framework for our results, indicating that NF-κB is directly required for activation of FasL transcription. Our work extends the results of Kasibhatla *et al*. by demonstrating that T cell activation signals work in a manner similar to DNA damaging agents in respect to FasL induction. This is interesting in that an NFAT site, located approximately 200 bp upstream from the transcription start site, was also identified as an important regulator of the FasL promoter.[74] Unlike etoposide, PMA/ionomycin treatment activates NFAT. Our results suggest that NFAT activation is not sufficient to activate the FasL promoter in the absence of NF-κB. Interestingly we have generated preliminary data demonstrating that both NF-κB and SP-1 are capable of binding to the putative NFAT site in EMSA assays (Cano and Scheinman, unpublished observations). The function of the NFAT site in the regulation of FasL promoter activity may be more complex than originally suspected. A second level of complexity comes from the study of transgenic mice in which the expression of the IκBα mutant has been directed to thymocytes. FasL expression was detected in peripheral T cells derived from these animals.[61] This would suggest that NF-κB may not be required for FasL expression in all T cell contexts however the extent to which the mutant IκBα inhibits NF-κB in mature T cells in this transgenic mouse has not been fully explored. Constitutive expression of FasL, as seen in Sertoli cells, would not be predicted to require NF-κB activation.

It is clear that there is no general role for NF-κB in the regulation of apoptosis but rather, NF-κB functions as a complex and cell specific regulator of apoptosis. In a study of Fas resistant T24 human bladder carcinoma cells, it was observed that ligation of Fas resulted in the activation of NF-κB while no such activation was observed in Jurkat T cells.[75] In a separate study, ligation of Fas in Jurkat T cells was shown to inhibit NF-κB activity, via a caspase-3-related protease mediated cleavage of relA and p50 thus inhibiting NF-κB activity early in the apoptotic process.[76] Another group has also recently reported that c-Rel expression prevents both TNFα and FasL mediated apoptosis in transformed lymphoid cells.[77] It is important to note in each of these cases that apoptosis, when mediated by the ligation of a death receptor, requires the interaction of an effector cell with a target cell. These data would suggests that the proapoptotic function of NF-κB is limited to a point upstream of Fas ligation, within the effector cell, and that NF-κB activity may interfere with later stages of the apoptotic process within the target cell, perhaps due to the ability of NF-κB to induce antiapoptotic genes such as cIAP and TRAFs.[43] In this regard, it will be interesting to see whether NF-κB functions in a similar fashion as a proapoptotic regulator in the CNS.

In the present study, we demonstrate, for the first time, that DEX mediated apoptosis is sensitive to the state of NF-κB activity. Inhibition of NF-κB results in a tenfold increase in the sensitivity of these cells to DEX (Figure 6 and 7). Previously, we have shown that GR and NF-κB interact to mutually inhibit each other. Thus one interpretation of this result in that by inhibiting NF-κB we have removed an inhibitor of GR function, allowing more GR to be available to induce apoptosis. We do not favor this hypothesis however as NF-κB must be induced to function, suggesting that release from IκBα is a prerequisite for interaction with GR. Indeed, we can only detect low levels of NF-κB DNA binding activity in nuclear extracts derived from uninduced T cell hybridomas (Figure 2 and data not shown). In addition, we have found that an excess of NF-κB must be present to physically inhibit GR.[63] A second interpretation of Figure 6 is that low levels of NF-κB activity, found in uninduced T cells, activate the expression of one or more genes which function to inhibit DEX mediated apoptosis. Indeed as mentioned above, several apoptosis inhibitors have recently been reported to be under NF-κB control and play an important role in protecting from TNFα induced apoptosis.[43,44,78] It will be interesting to see what role these genes might play in the regulation of glucocorticoid mediated apoptosis.

These results supply a physiological interpretation to our previous discovery that GR and NF-κB are mutually inhibitory and demonstrates a potential physiological role for NF-κB as a regulator of apoptosis in the immune system. We hypothesize that during thymic selection, NF-κB protection from glucocorticoid mediated apoptosis may represent a mechanism by which thymocytes survive positive selection. In order to induce apoptosis, GR must induce genes (such as IκBα) to block apoptosis inhibitors as well as to induce genes to promote apoptosis itself. After differentiation is completed, and the mature T cell is exported to the periphery, the role of NF-κB then changes to promote the linkage of T cell activation with eventual deletion through induction of proapoptotic molecules such as FasL. Most likely, other proapoptotic genes will be identified as NF-κB responsive in addition to FasL. Given that different forms of NF-κB are expressed at different points in T cell differentiation, it is intriguing to speculate that different forms of NF-κB may responsible for antiapoptotic and proapoptotic functions. As the mutant IκBα inhibits all forms of NF-κB, we were unable to address this issue in the present study. Further experiments will need to be performed in primary T cells to identify which forms of NF-κB are responsible for these functions.

Materials and Methods

Cell culture

DO11.10 and 2B4.11 T cell hybridoma cultures were maintained in Dulbecco's Minimal Essential Media (DMEM; Gibco BRL) supplemented with 10% FBS (Gemini Bio-Products) and Penicillin-Streptomycin (50 units/ml and 50 mg/ml respectively; Gibco BRL). T cell hybridomas were kept at a concentration of 500 000 cells/ml or less. Stably transfected cells were further supplemented with 450 μg/ml of G418 Sulfate (Gemini Bio-Products) to select for transgene expression. Cells were maintained at 37°C and 5% CO_2. Cells were activated either by treatment with 5 ng/ml PMA and 500 ng/ml Ionomycin (Sigma) or by treatment with anti-CD3 for varying periods of time. For anti-CD3 treatment, 96-well plates were coated with 100 μl 5 μg/ml anti-CD3 (in PBS pH 9.0) overnight and washed with PBS (pH 7.0) before use.

Transfection of cell lines

DO11.10 and 2B4.11 T cell hybridomas were stably transfected by electroporation. An aliquot of 2×10^6 cells were suspended in 500 μl of RPMI 1640 media (Gibco BRL) supplemented with MEM Non-Essential Amino Acids (Gibco BRL) and 1 mM MEM Sodium Pyruvate (Gibco BRL). Using 0.4 cm electroporation cuvettes (Invitrogen) samples were electroporated at a capacitance of 960 μF and 250 volts with 5 μg of pCI-neo (Promega) in the presence or absence of either 20 μg of pCMV4-IκBαS32/36A or 20 μg of pCMV4-FLAG-IκBαS32/36A (gifts of D. Ballard, Vanderbilt University). Cells were then removed and placed into 5 ml of supplemented DMEM (as above) for 72 h then fed with G418 supplemented DMEM (as above) for further culture. After several days clones were isolated by dilution of the cells and plating into 96 well plates, then visually scanning for wells that contained single cells which were then cultured. Individual colonies were grown and frozen in aliquots before use.

Electrophoretic mobility shift assays (EMSA)

Extracts for EMSA were prepared from 10^7 cells. T cell hybridomas were initially lysed by dounce homogenization in buffer A (10 mM Tris [pH 7.9], 0.75 mM spermine, 0.15 mM spermidine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl) and protease inhibitors (100 mg/ml PMSF, 100 μM leupeptin, 10 μM pepstatin A). The lysed cells were spun down (12 000 × *g*, 10 min, 4°C) and the supernatant removed. The nuclear pellet was quickly washed in 1 ml buffer A to remove residual cytoplasmic extract, spun 3 min as above, and then resuspended in 50 μl buffer C (20 mM Tris [pH 7.9], 0.75 mM spermine, 0.15 mM spermidine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 20% glycerol) plus protease inhibitors. The volume was measured and NaCl added to a final concentration of 0.4 M while mixing. The nuclei were extracted on ice for 15 min with occasional mixing. The nuclei were then pelleted (13 000 × *g*, 10 min, 4°C) and the supernatant was removed as nuclear extract. Nuclear and cytoplasmic extracts were aliquoted and quick frozen using dry ice/ethanol bath. Protein concentrations were measured by method of Bradford with Bio-Rad dye reagent used according to the manufacturer's instructions.[79]

DNA binding reactions were performed incubating 2–5 μg of nuclear extract with 0.5 μg poly (dI-dC) in binding buffer (5 mM Tris [pH 7.0], 50% glycerol, 0.5 mM DTT, 0.25 mM EDTA) for 10 min at room temperature. Approximately 10 000 c.p.m. of ^{32}P-labeled DNA probe was then added and allowed to bind for 30 min. The reaction was then loaded onto a 5% native acrylamide TGE (25 mM Tris, 190 mM glycine, 1 mM EDTA) gel. After gel electrophoresis, the gels were dried and data visualized by Phosphor Imager.

DNA probes and unlabeled competitors were prepared by annealing oligonucleotides and performing fill-in reactions with the Klenow fragment of DNA polymerase I (GIBCO) according to the manufacturer's instructions and as described.[80] Our NF-κB probe derived from the FasL gene including the putative NF-κB site is CAGAAAATTGTGGGCGGAAACTTCCAGGGGTTTCGTC and corresponds to the region spanning from −291 to −259 relative to the translation start site.[68] The NF-κB binding site competitor is derived from the region I enhancer of the H-2K^b gene with the sequence CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC. A double point mutant of this site has the sequence CAGGGCTG**C**GGATTCCC**G**ATCTCCACAGTTTCACTTC.[80] Mutated bases are in bold and underlined.

Western blots

Cytoplasmic extracts were prepared as described above. A volume of extract corresponding to 20 μg protein was size separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose by electro-blotting in 25 mM Tris base, 0.2 M glycine, 20% methanol at 100V for 1 h at 4°C. The nitrocellulose blot was blocked for 1 to 2 h in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) plus 10% dried milk. FLAG-probe (D8) antibody (Santa Cruz) was diluted 1 : 2,000 in TBST plus 10% dried milk for 1 h at room temperature with gentle shaking. The blot was washed five times for 5 min each in TBST. An HRP conjugated anti-rabbit IgG antibody (Promega) was diluted 1 : 10 000 in TBST plus 10% dried milk and the blot incubated with this secondary antibody for 1 h at room temperature with gentle shaking. The blot was washed five times as described above. The secondary antibody was visualized by electrochemiluminescence (ECL, Amersham) according to the manufacturers instructions and recorded on Kodak XAR5 film.

Extraction and purification of RNA and RT–PCR

Total RNA was prepared by the acidified phenol method as described.[69] The mRNA was converted to cDNA utilizing SuperScript II RT (Gibco BRL) with oligo dT (Gibco) as the primer as directed by the manufacturer. Two μl of the cDNA was used as template for PCR using primers for mouse FasL: TTCTGGGTAGACAGCAGTGC-CACTTCATC and CAGTTTCGTTGATCACAAGGCCACCTTTC or β-actin: 5′ GATGCATTGTTACAGGAAGT and 3′ TCATACATCT-CAAGTTGGGGG. Reaction conditions for FasL amplification were 35 cycles of, melting at 94° for 30 s, annealing at 65° for 30 s, and extension at 72° for 45 s. Reaction conditions for β-actin amplification were 12 cycles of melting at 94° for 30 s, annealing at 48° for 30 s, and extension at 72° for 45 s. PCR products were separated on a 1% agarose gel with ethidium bromide and visualized with UV and recorded by Polaroid and scanned.

Apoptosis and cell viability assays

Apoptosis was assayed using the acridine orange/ethidium bromide double dye technique.[71] Sample cells were spun down (900 × *g*, 5 min) and washed with PBS. Two μl of dye mix (100 μg/ml acridine orange (Sigma) +100 μg/ml ethidium bromide in PBS) was placed in the bottom of a 12 × 75-mm glass tube and 25 μl of cell suspension was added to the tube and mixed by hand. Ten μl of this mixture was placed on a microscope slide and covered with a 22-mm^2 cover slip. Samples were examined with a 40× dry objective using epi-illumination and a filter combination suitable for observing

fluorescein. One hundred cells were counted per sample and the number of cells in each of the following four states was recorded: VN – viable normal (bright green chromatin with organized structure), VA – viable apoptotic (bright green chromatin that is condensed or fragmented), NVN – non-viable normal cells (bright orange chromatin with organized structure), NVA – non-viable apoptotic (bright orange chromatin that is condensed or fragmented). Per cent apoptosis was then calculated using the following formula: VA+NVA/100=% apoptosis.

Cell viability was assayed using trypan blue exclusion. A 100 μl aliquot of cells was taken and mixed with 100 μl of trypan blue solution (0.1% in PBS). A 10 μl aliquot was placed on a hemocytometer and number of viable cells (clear) and non-viable cells (dark blue) were counted. Results were expressed as a percentage of cells counted.

^{51}Chromium labeling and release assay

Fas expressing L1210-Fas cells to be used as targets in ^{51}Cr release assays were suspended at $1-5\times10^6$ in 100 μl of RPMI 1640 medium with 5% heat inactivated FBS (R5) to which 100 μCi of ^{51}Cr in R5 were added. The cells were then incubated for 90 min at 37°C, washed twice in R5, resuspended in 10 ml of R5, and incubated at 37°C for 30 min. They were washed once more, resuspended in R5, and counted. The labeled cells (10^4 in 100 μl) were placed in individual wells of 96-well V-bottomed microtiter plates that contained various concentrations of effector cells in triplicate in 100 μl of R5. The plates were centrifuged ($50\times g$; 5 min) to establish cell contact and incubated at 37°C for 18 h prior to harvest. At the end of the incubation period, the plates were centrifuged at $100\times$ g for 10 min and 100 μl of cell-free supernatant was collected from each well. Radioactivity in supernatants were measured in a gamma counter. Per cent specific release was calculated by the following formula:

$$\%\ \text{specific}\ ^{51}\text{Cr release} = \frac{\text{c.p.m.}_{\text{exp}} - \text{c.p.m.}_{\text{spont}}}{\text{c.p.m.}_{\text{max}} - \text{c.p.m.}_{\text{spont}}} \times 100$$

Maximal release (c.p.m.$_{max}$) was determined from supernatants of cells that were subjected to 1% Triton-X. Spontaneous release (c.p.m.$_{spont}$) was determined from target cells incubated without added effector cells.[81]

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