Cell Death and Differentiation (2003) 10, 1032–1044

www.nature.com/cdd

Antiapoptotic function of NF-κB in T lymphocytes is influenced by their differentiation status: roles of Fas, c-FLIP, and Bcl-x_L

AL Mora[1,5], RA Corn[1,2,6], AK Stanic[1,6], S Goenka[1,6], M Aronica[3,7], S Stanley[1], DW Ballard[1], S Joyce[1] and M Boothby*[,1,4]

[1] Department of Microbiology & Immunology, Vanderbilt University Medical School, Nashville, TN 37232-2363, USA, and
[2] Meharry Medical College, TN, USA
[3] Allergy, Pulmonary, & Critical Care Medicine Division, Department of Medicine, Vanderbilt University Medical School, Nashville, TN 37232-2363, USA
[4] Rheumatology Division, Department of Medicine, Vanderbilt University Medical School, Nashville, TN 37232-2363, USA
* Corresponding author: M Boothby, Department of Microbiology and Immunology, Vanderbilt University Medical School, Nashville, TN 37232-2363, USA. Tel: 615 343 1699; Fax: 615 343 7392; E-mail: mark.boothby@mcmail.vanderbilt.edu
[5] Current address: Department of Medicine, Emory University Medical School, Atlanta, GA, USA
[6] Equal contributions
[7] Current address: Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

Received 10.6.02; revised 28.2.03; accepted 5.3.03
Edited by ME Peter

Abstract

Inducible protection from apoptosis *in vivo* controls the size of cell populations. An important question in this respect is how differentiation affects mechanisms of apoptosis regulation. Among mature T lymphocytes, the NF-κB/Rel transcription factors are coupled to receptors that control cell population sizes by concurrently regulating survival and multiplication. In the present study, we used a transgenic inhibitor of NF-κB/Rel signaling to investigate the role of this pathway in proliferation and death of mature T cells *in vivo*. The results indicate that NF-κB integrates two critical yet distinct molecular pathways preventing apoptosis affected by the death receptor Fas, coordinately regulating levels of FLIP and Bcl-x_L in primary T cells. Surprisingly, NF-κB blockade preferentially impacted naive as compared to memory T cells. The Fas/FasL pathway was linked to these findings by evidence that the abnormalities imposed by NF-κB inhibition were ameliorated by Fas deficiency, particularly for the $CD4^+$ lineage. Moreover, levels of an inhibitor of Fas-mediated apoptosis, c-FLIP, were diminished in cells expressing the transgenic inhibitor. NF-κB was also linked to T cell survival *in vivo* by mediating induction of Bcl-x_L: restoration of Bcl-x_L levels reversed the preferential deficit of naive T cells, differentially impacting the CD4 and CD8 subsets. These results show that promoting survival and effective multiplication are central roles for NF-κB in T lymphoid homeostasis *in vivo*, but this effect and its underlying mechanisms are influenced by the developmental state of the lymphocyte.
Cell Death and Differentiation (2003) **10,** 1032–1044. doi:10.1038/sj.cdd.4401257

Keywords: transcription factors; T lymphocytes; homeostasis; Bcl-x_L; FLIP

Introduction

Signal-induced regulation of apoptosis makes key contributions to the determination of cell population size during homeostasis, growth, differentiation, and tissue remodeling. Cellular differentiation may take place concurrent with the growth or maintenance of tissues, so that the influence of developmental state on the function of specific molecular mechanisms of cell survival *in vivo* is a fundamental question in understanding these processes. Lymphoid cell populations represent an attractive model for studying the effect of differentiation state on mechanisms of apoptosis regulation. The immune system establishes and maintains homeostasis, that is, normal sizes and subset compositions, for pools of differentiated $CD4^+$ and $CD8^+$ lineages of T cells.[1,2] The size of each mature T lymphoid population results from thymic production rates, signal-dependent survival of resting and activated T lymphocytes, the multiplication and death of these cells during immune responses, and differentiation into a memory-phenotype subset derived from naive T cells.[1–6] Moreover, T cells can sense niche availability in peripheral compartments so as to regulate the pool of cycling T cells by limiting or increasing clonal expansion.[7–10] Thus, an additional mechanism which can contribute to restoration of normal T cell numbers has been termed homeostatic proliferation. However, the molecular mechanisms that establish and maintain normal T cell numbers within these developmental subsets are not understood.

Survival of naive T cells depends on the presence of an appropriate ligand for the T cell antigen receptor (TCR).[11–14] The NF-κB/Rel protein family provides critical integration between signal transduction and transcription pathways activated in T cells after engagement of the TCR, costimulatory molecules, and cytokine receptors.[15–17] Thus, it has been important to determine the role of NF-κB in T cell survival, death, and homeostasis *in vivo*. However, this goal has been complicated by functional redundancy among members of the family, and no knockout of these genes has been shown to play a cell-intrinsic role in determining lymphoid population size or apoptosis rates *in vivo* (reviewed in Ghosh *et al.*[15]). To bypass potential functional redundancy among NF-κB/Rel proteins and investigate the role of this family in the death of T cells, we generated transgenic mice expressing a mutant form

of IκB specifically in T lineage cells.[18] Expression of this mutant protein, termed IκBα(ΔN), functioned as an effective repressor of NF-κB/Rel signaling in T lineage cells. Although thymic precursors of the mature T cell subsets were virtually normal in these mice, interference with NF-κB induction led to a substantial reduction of the $CD4^+$ subset and a dramatic decrease of $CD8\alpha\beta^+$ mature T cells in the periphery.[18,19] In addition, our *in vitro* analyses indicated that IκBα(ΔN) T cells exhibit increased apoptosis after TCR crosslinking with anti-CD3.[18] These findings suggested that mechanisms operative in the periphery to be responsible for the dramatic deficits in the population of mature T cells because of NF-κB blockade. Alternatively, however, a decrease in the pool of TCR^{hi} CD8 single positive thymocytes and other evidence of decreased positive selection when NF-κB is inhibited raise the possibility that a decreased rate of T cell production in the thymus exclusively accounts for the deficit of mature T cells.[18,20] Furthermore, the signaling requirements for survival differ between naive lymphocytes and their differentiated progeny, memory-phenotype T cells (reviewed in Dutton *et al*.[11]; Boothby *et al*.[21]). Thus, naive but not memory T cells require the antigen receptor to survive, whereas signaling through hematopoietin receptors sharing the common gamma (γc) chain contributes differentially to the homeostatic expansion of naive *versus* memory-phenotype T cells.[21–24] While these observations suggest that survival and thus homeostasis are maintained through the integration of signals from the TCR and collaborating receptors, very little is known about the differences in programming between naive and memory-phenotype subsets in terms of the signaling pathways or transcription factors essential for preventing T cell apoptosis and allowing multiplication in the periphery.

In the present study, we have dissected the role of NF-κB in death and homeostasis of differentiated subsets of T cells. Blocking NF-κB signaling led to major defects of T cell multiplication *in vivo.* Surprisingly, however, it was found that the expansion of naïve T cells was preferentially dependent on NF-κB signals as compared to memory-phenotype cells. This differential role of NF-κB was associated with a progressive distortion of the balance between these two subsets. Two apoptosis-related mechanisms were linked to these homeostatic defects in IκBα(ΔN) mice. First, the population of naive T cells *in vivo* was decreased in association with attenuated Bcl-x_L inducibility, and CD4 T cell numbers were restored in an epistasis analysis using constitutive Bcl-x_L expression in T cells. The data also indicated that NF-κB proteins play a role in the control of T cell AICD *in vitro,* and Fas expression contributes to the deficits of mature T cells *in vivo*, particularly for $CD4^+$ cells. This finding was correlated with decreased induction of FLIP, an inhibitor of death receptor signals, in naive T cells whose NF-κB induction is inhibited. Taken together, the data show that NF-κB inducibility in normal T lymphocytes plays a critical role in maintaining their numbers *in vivo* by promoting the survival of naive T cells, but highlight that the differentiated state within the T lineage determines the degree to which specific molecular mechanisms (NF-κB, Bcl-x_L) regulate cell survival. The findings further suggest that mechanisms promoting the survival of naive T cells include a dependence on NF-κB for coordinate induction of antiapoptotic factors as T lymphocytes reside in their microenvironment, leading to resistance to Fas-induced death.

Experimental Procedures

Mice

IκBα(ΔN) transgenic mice (Tg), in which expression of a stable inhibitor of NF-κB/Rel activation[25] is targeted to the T cell lineage using the lck promoter and CD2 locus control region, have been described previously.[18] IκBα(ΔN) transgenic mice on a BALB/c (H-2d; BC≥6) background were bred with DO.11.10 TCR transgenic BALB/c mice, and those on a C57BL/6 background (H-2b; BC≥5) were bred with lines for the previously described 2C TCR transgene.[26] Mice carrying the autosomal recessive lpr mutation on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). IκBα(ΔN) transgenic mice on BALB/c and C57BL/6 backgrounds were bred with transgenic mice constitutively expressing Bcl-x_L (provided by Dr. Craig Thompson;[27]) and crossed onto BALB/c or B6 backgrounds. IκBα(ΔN), DO-11.10-TCR double-transgenic mice were subsequently bred with Bcl-x_L, DO-11.10-TCR double-transgenic mates to generate DO-11.10-positive littermates expressing all four combinations of IκBα(ΔN) and Bcl-x_L transgenes. Genotyping of the DO.11.10, 2C TCR, Bcl-x_L transgenes, and lpr mice was performed by PCR. Mice were maintained in microisolator cages under SPF conditions, and used in accordance with applicable regulations with institutional approval.

Antibodies, fluorescence reagents, and flow cytometric analyses

Biotinylated and fluorochrome-conjugated antibodies against CD8 (biotin, FITC or r-PE), CD4 (biotin, FITC or r- PE), TCR Vβ8.1/2 (r-PE), CD44 (FITC), CD62L (FITC), CD69 (FITC), Ly6C (biotin), Thy1.2 (biotin), and activated caspase-3 (r-PE) as well as streptavidin-PerCP were obtained from B-D PharMingen (Mountain View, CA, USA). Anticlonotypic antibodies against the DO-11.10 and 2C TCRs were purified from the appropriate hybridomas (KJ1-26 and 1B2, respectively) and biotinylated. Magnetized microbeads for magnetic cell sorting (strepavidin-, anti-CD8, -MHC-II, and -CD62L-conjugated) were obtained from Miltyeni BioTec (Auburn, CA, USA), 7-actinomycin D (7-AAD) from Molecular Probes (Eugene, OR, USA). Most flow cytometry was performed as described previously,[18,19] while Bcl-x_L expression was determined by flow cytometry as described[28] and the levels of activated caspase-3 were measured according to the manufacturer's instructions.

Cell and preparative sorting

Single cell suspensions were prepared from thymus, spleen or lymph nodes as described previously.[18] For preparative cell sorting, $Thy1.2^+$ lymphocytes from spleen and lymph node of wild-type (WT) and IκBα(ΔN) transgenic mice were obtained by positive selection using streptavidin-magnetic beads (Miltyeni BioTec). Cells were incubated with biotinylated

anti-Thy1.2 antibody (15 min at 4°C), washed with bovine serum albumin (0.5% w/v in PBS), and incubated for 10 min at 4°C with streptavidin-magnetic beads. After 10 min, cells were washed extensively and cells bound to the anti-Thy1.2 antibody were applied to a column and subjected to positive selection using a magnet (Miltyeni BioTec). Positively selected cells were stained with anti-CD44 (FITC) and SA-PerCP. Lymphocytes were then sorted into Thy1.2$^+$, CD44^{lo} and Thy1.2$^+$, CD44^{hi} populations. Magnetic sorting according to manufacturer's instructions was used to separate T cells according to the levels of CD62L expression.

Adoptive transfers of TCR transgenic cells

For transfer of CD4$^+$ T cells from DO11.10 TCR transgenic mice, single cell suspensions prepared from lymph nodes of DO11.10, DO11.10 :: IκBα(ΔN), DO11.10 :: Bcl-x_L, and DO11.10 :: IκBα(ΔN) :: Bcl-x_L mice were stained with anti-CD4, biotinylated anticlonotypic mAb KJ1-26, and streptavidin-PerCP. CD4$^+$, KJ1-26$^+$ cells (2×10^6 per recipient) were transferred into nonirradiated BALB/c mice. One day later, recipient mice were immunized with an emulsion consisting of equal volumes of OVA (2 mg/ml in sterile PBS) and CFA H37 Ra (Difco, Detroit, MI, USA), for which 200 μl of emulsion was divided among three sites (each flank and at the base of the tail). After 7 days, inguinal, mesenteric, and periaortic lymph nodes were harvested and analyzed. For measurement of CD8 cell multiplication *in vivo*, pooled cells from spleen and lymph nodes of WT and IκBα(ΔN) transgenic mice (ages 6 months) positive for the 2C TCR transgene were transferred into lethally irradiated (750R) BALB/c mice (10^6 CD8$^+$ 1B2$^+$ cells per recipient). After 4 days, splenocytes harvested from recipient mice were counted, stained with anti-CD4, anti-CD8, and the clonotypic mAb against 2C TCR (1B2), and analyzed by flow cytometry. For analysis of induced cell death after allostimulation *in vivo*, splenocytes harvested from recipient mice 2 days after adoptive transfer were counted and stained with anti-CD8 or anti-B220 mAb. Cells were resuspended in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and dead cells were detected by Annexin V (Pharmigen) and 7-AAD. For parking experiments, cells from spleen and lymph node of WT and IκBα(ΔN) transgenic mice positive for the 2C TCR transgene were transferred into irradiated C57BL/6 mice (3×10^6 CD8$^+$ 1B2$^+$ cells per recipient). After 1 and 7 days, splenocytes were harvested from recipient mice, counted, stained with anti-CD4, anti-CD8, and 1B2 mAbs, and analyzed by flow cytometry. For adoptive transfer of TCR transgenic CD44^{lo} and CD44^{hi} cells, spleen and lymph node of WT and IκBα(ΔN) transgenic mice were stained and sorted into CD8$^+$CD44^{lo} and CD8$^+$CD44^{hi} cells. CD8$^+$ CD44^{lo} or CD8$^+$CD44^{hi} cells (106 per recipient) were transferred into syngeneic RAG2-deficient mice (Jackson Lab) and followed by weekly phlebotomy and FACS analyses. After 7 weeks, spleens and lymph node cells harvested from recipient mice were counted, stained with anti-CD8 and anti-CD44, and analyzed by flow cytometry.

Retroviral transduction and AICD assays

IκBα(ΔN) cDNA was inserted into the bicistronic MSCV2.2-IRES-GFP (GFP-RV).[29] Retrovirus-containing supernatants were collected 48 h after transfection of ΦNX ecotropic packaging cells, and centrifuged (1 h, $10\,000 \times g$) with ConA-activated splenocytes as described.[30] Cells cultured 2 days in complete medium and IL-2 were washed and then stimulated with plate-bound anti-CD3. Cell viability was determined from the percentages of 7-AAD$^+$ cells in the CD4$^+$ and CD8$^+$ gates. TUNEL assays were performed as described.[18] Control experiments documented that transduction with IκBα(ΔN) led to a 95% reduction in NF-κB induction in the GFP$^+$ population of T cells as compared to empty GFP-RV.

Northern blot analyses

Splenic and lymph node T lymphocytes were depleted of B cells by chromatography through nylon wool columns as described previously.[18,19] T cells were plated (2.5×10^6 cells/ml) and cultured overnight at 37°C in the presence of plate bound anti-CD3 (10 μg/ml) plus anti-CD28. Total cellular RNA was isolated using TriZol reagent according to the manufacturer's instructions (Life Technologies, Bethesda, MD, USA). After resolving RNAs by electrophoresis on formaldehyde agarose gels, nucleic acids were detected and band intensities were quantitated by hybridization with radioactive cDNAs and phosphorimaging.

Immunoprecipitation, Western blot, and gel mobility shift analyses

For measurements of IκBα, sorted CD44^{lo} and CD44 T cells were used to prepare whole cell extracts and perform immunoprecipitations as described previously.[18] Precipitated proteins were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and processed as described.[18] For gel mobility shift analyses, nuclear fractions were prepared from single cell suspensions (thymocytes and preparatively sorted CD44^{lo} and CD44^{hi} CD8$^+$ lymphocytes) and gel mobility shift assays were performed using radiolabeled κB-pd probe as described.[18] For analysis of FLIP expression, T cells were purified by depletion using anti-MHC class II antibody attached to magnetic beads (MACS). T cell-enriched preparations were sorted into naïve and memory-phenotype T cells using anti-CD62L antibody attached to MACS beads. Extracts from cells cultured in the presence of 1 μg/ml plate-bound anti-CD3 and 2.5 μg/ml anti-CD28 were subjected to SDS-PAGE, and resolved proteins were probed using Dave-2 anti-FLIP mAb (Alexis Biochemicals) after transfer to PVDF membranes. Relative loading was assessed on stripped blots reprobed with anti-cyclophilin B (Alexis Biochemicals).

Proliferation, BrdU incorporation, and RNA synthesis assays

Splenocytes were plated (2×10^5 cells per 100 μl of media) in microtiter wells previously incubated overnight with PBS or anti-CD3 mAb (10 μg/ml; clone 2C11, PharMingen). Triplicate samples were cultured for 48 h at 37°C in the presence of medium, plate-bound anti-CD3, or Con A (2.5 μg/ml). Tritiated thymidine (1 μCi in 100 μl of media) or, as in,[31] tritiated uridine

(0.25 μCi in 100 μl of media), was added to each well for the final 8 h before determination of radioisotope incorporation into DNA or RNA, respectively. For short-term measurements of S-phase entry rates, cells from spleen and lymph node were cultured for 40 h in the presence of Con A (2.5 μg/ml) and then treated for 1 h with 5-bromo-2′-deoxyuridine (BrdU; 100 μM). BrdU-positive $CD4^+$ and $CD8^+$ cells were measured as described.[32] In brief, after staining with either anti-CD4- or anti-CD8-rPE, cells were permeabilized with 95% EtOH, fixed with paraformaldehyde, treated (10 min at 22°C) with 50 U DNase I (Boehringer-Mannheim Biochemicals, Indianapolis, IN, USA), stained with a FITC-conjugated mAb against BrdU (Beckton-Dickson, Mountain View, CA, USA), and analyzed by flow cytometry.

Results

Cell-intrinsic NF-κB signaling is required for T cell multiplication *in vivo*

The number of both $CD4^+$ and $CD8^+$ peripheral T cells is significantly reduced in mice whose T cells have been subjected to inhibition of NF-κB inducibility.[18,28,33–35] One potential explanation for this observation is that the rate of T cell production in the thymus may be diminished when NF-κB is blocked.[18,20,35,36] However, the populations of T lymphocytes normally exhibit an intrinsic capacity to achieve normal numbers in a manner independent of the cell input.[4,5] We therefore hypothesized that an enhanced susceptibility to apoptosis and a diminished ability to multiply in the intact animal were key mechanisms giving rise to the reduced population of mature T cells.

To determine whether or not the ability to induce NF-κB is essential for homeostasis of mature T cells *in vivo*, we tested if mechanism(s) regulating mature T cell population sizes *in vivo* were affected by the IκBα(ΔN) transgene. We first measured ability of $CD4^+$ T cells to multiply at normal rates in the intact animal when subject to inhibition of their NF-κB induction. After transfer of equal numbers of WT or mutant $CD4^+$ cells bearing a unique antigen receptor (DO11.10 TCR) into recipients lacking this marker but bearing normal numbers of T cells, the frequency of antigen-specific T lymphocytes at their site of multiplication (draining lymph node) was measured. WT T cells underwent significant population expansion, whereas those bearing the IκBα(ΔN) transgene did not (Figure 1a). To perform similar measurements of clonal

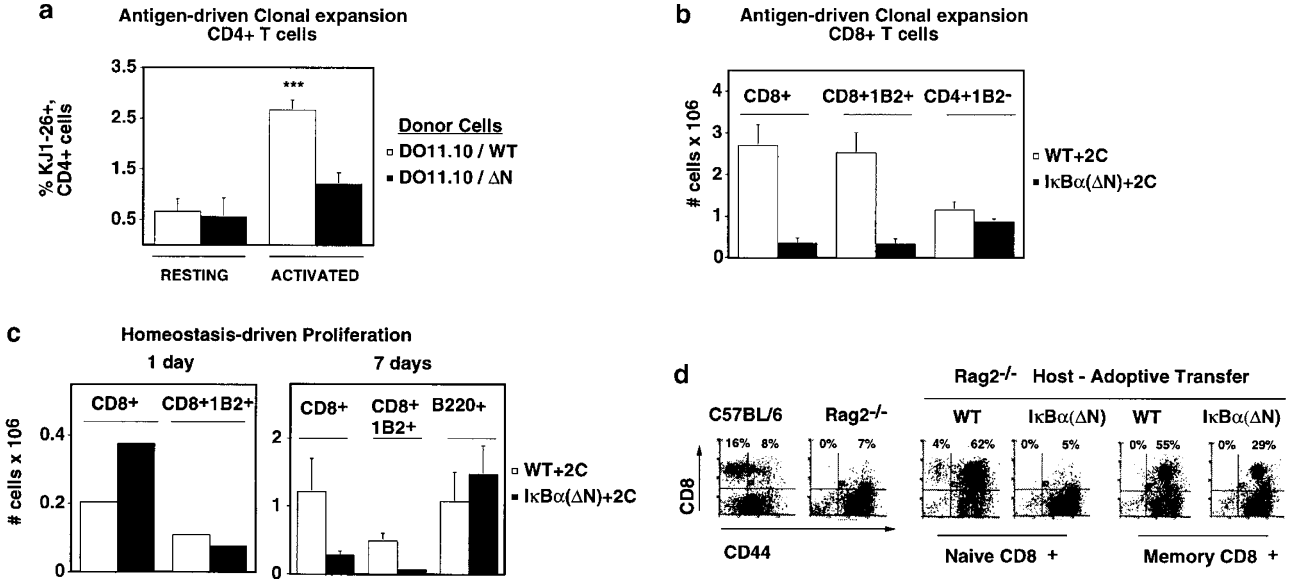

Figure 1 Diminished clonal expansion *in vivo* of T cells with defective NF-κB/Rel signaling. (**a**) Inhibition of NF-κB attenuates clonal expansion of $CD4^+$ T cells. Equal numbers of $CD4^+$ T cells, as determined by flow cytometry, were prepared from BALB/c-DO11.10 TCR transgenic mice expressing IκBα(ΔN), or littermates with normal NF-κB signaling and transferred into naive syngeneic mice. Draining lymph node cells from mice immunized the next day with OVA323–339 peptide in CFA ('Activated') were harvested six days later, and the frequency of donor-derived T cells was determined by staining with the anti-clonotypic mAb KJ1-26 and CD4 (***, $P<0.001$ compared to each other sample set). Under these conditions, other work has failed to detect an abnormal localization of IκBα(ΔN) T cells to nonlymphoid tissue sites.[19,74,75] As a control, frequencies of cells in the KJ1-26$^+$CD4$^+$ gate were measured without immunization ('RESTING'). (**b**) Defective alloreactive expansion. Lymphoid cells from H-2^b, 2C TCR transgenic mice bearing or lacking the IκBα(ΔN) transgene were transferred into irradiated BALB/c recipients (10^6 1B2$^+$ CD8$^+$ cells/mouse). After 4 days, viable splenocytes were enumerated, stained with CD8 and anticlonotypic mAb against the 2C TCR (1B2), and analyzed by FACS. Cell numbers were calculated from the percentage of cells positive for the indicated markers. Data represent the mean determined in three independent experiments (open bars, WT-2C$^+$ BALB/c recipients (n=5); filled bars, IκBα(ΔN)-2C$^+$ BALB/c recipients (n=7)) (**c**) Defective homeostatic expansion. Cells from 2C mice, as described in (**b**), were transferred into irradiated syngeneic recipients (3×10^6 1B2$^+$CD8$^+$ cells per mouse). After 1 and 7 days, viable splenocytes were enumerated and analyzed by FACS after staining with CD8, 1B2, and B220 mAbs. Cell numbers were calculated as in (**b**). Data are the mean obtained from four animals in a representative experiment. (**d**) NF-κB preferentially affect clonal expansion of naïve T cells. Lymphoid cells from 2C TCR transgenic mice (6 months old) bearing or lacking the IκBα(ΔN) transgene were sorted into CD8$^+$CD44^{lo} and CD8$^+$CD44^{hi} cells and transferred into syngenic RAG2-deficient mice (10^6 cells/mouse). After 7 weeks, cells from spleen were counted and analyzed by FACS after staining for the indicated markers. Representative profiles from the viable cell gate in the spleens of control mice (WT C57BL/6 and Rag2$^{-/-}$ mice without transferred cells) and recipient mice are shown. Numbers indicate relative percentage of positive cells within a quadrant; the few CD8^{lo} cells observed in Rag2$^{-/-}$ mice are presumed CD8α$^+$ dendritic cells. The observed increase in CD44 expression in CD8$^+$CD44^{lo} cells during homeostatic expansion is as previously described[41,42]

expansion for the CD8 lineage of T cells, equal numbers $CD8^+$ cells bearing a distinct antigen receptor (2C TCR) were transferred into recipients whose combination of the MHC class I molecule L^d and a self-derived peptide[26,37] strongly drive T cell proliferation via stimulation of this TCR. WT $CD8^+$ T cells bearing the 2C TCR ($1B2^+$) underwent clonal expansion in response to the foreign MHC molecule (L^d). In contrast, the multiplication of clonotype-positive cells under these conditions was blocked by the transgenic inhibitor, IκBα(ΔN) (Figure 1b). One mechanism which can help maintain normal numbers of T cells is that they can undergo MHC interaction-dependent, antigen-independent proliferation in lymphopenic environments. This process, homeostatic proliferation, is more rapid for CD8 as compared to CD4 T lymphocytes.[7–10] To analyze whether defective maintenance of homeostatic proliferation contributes to the lack of T cells observed in IκBα(ΔN) mice, we transferred marked $CD8^+$(H-2^b) T cells into irradiated syngeneic (H-2^b) recipients. Enumeration of donor-derived CD8 T cells 1 week after transfer showed a dramatic reduction in the clonal expansion of T cells expressing the IκBα(ΔN) transgene (Figure 1c). We conclude that the NF-κB/Rel signaling pathway regulates multiplication driven by antigen receptor stimulation for both the CD4 and CD8 lineages of T cells in the intact animal.

For the CD4 and CD8 lineages of T lymphocyte, achieving and maintaining normal-sized populations involves both naive T lymphocytes and their differentiated, memory-phenotype descendants.[5,11,38,39] These subsets differ with respect to their MHC-peptide and cytokine requirements for survival and proliferation,[13,21,24,40] but the signaling or transcriptional mechanisms that distinguish regulation of naïve T cell apoptosis or multiplication from memory-phenotype cells are unknown. To test whether inhibition of NF-κB differentially affects naive and memory-phenotype T cell subsets, we analyzed their proliferation after transfers of each population into lymphopenic mice. As the multiplication rate of $CD4^+$ cells would be too slow for such analyses, we focussed on the $CD8^+$ lineage of T cells. Prior to transfer, levels of CD44 were used to distinguish between naïve ($CD44^{lo}$) and memory-phenotype ($CD44^{hi}$) $CD8^+$ T cells from TCR transgenic mice. After transfer, naive cells undergoing homeostatic expansion became $CD44^{hi}$ as previously reported.[41,42] Nonetheless, donor $CD44^{lo}$ cells expressing the IκBα(ΔN) transgene had a far more severe reduction in their expansion capacity than their $CD44^{hi}$ IκBα(ΔN)-expressing counterparts when measured 7 weeks after transfer (Figure 1d). Thus, transferred IκBα(ΔN) $CD8^+CD44^{lo}$ cells expanded and accumulated four to 10-fold less than control cells in spleen and lymph node of host mice, respectively. In contrast, the prevalence of transgene $CD8^+CD44^{hi}$ cells 7 weeks after transfer was similar to WT controls in spleen, and only halved in lymph nodes. This difference was also observed during weekly monitoring of CD8 cells circulating in the blood during weeks 1–6 (data not shown).

Based on these findings, we reasoned that the population deficit of naive T cells in IκBα(ΔN) mice should become progressively more severe than that of memory-phenotype cells, thereby leading to a distortion of the normal ratio of these subsets over time. Accordingly, we measured the frequency of CD44 hyperexpression among resting $CD4^+$ and $CD8^+$ T cells at 2, 4, 6 and 8 months of age.[43–46] The $CD44^{hi}$ population size in IκBα(ΔN) mice was nearly normal (cells numbers were 50–90% than those in WT littermates, data not shown), but these cells prematurely dominated the $CD4^+$ and $CD8^+$ subsets, so that by 6 months of age most $CD8^+$ T cells were small, resting, and memory-phenotype (Figure 2a, b). Analogous changes were evident when using other markers of the memory-phenotype state of differentiation (CD45RB, CD62L, and Ly-6C; data not shown), whereas other cell surface markers (CD25 and CD69) were characteristic of memory-phenotype rather than activated T cells. The alternative explanation for this differential impact on naive T cells and their memory-phenotype descendants is that there is selection *in vivo* against T cells which expressed high levels of IκBα(ΔN). If cells with the lowest expression of transgene-encoded protein were best able to become memory-phenotype cells after MHC-peptide encounters, this mechanism would lead a dominance of $I\kappa B\alpha(\Delta N)^{lo}$, $CD44^{hi}$ T cells because of longer survival and the kinetics of cycling in the $CD44^{hi}$ population relative to $CD44^{lo}$ cells.[38,47] This explanation predicts that levels of the epitope-tagged IκBα and NF-κB inhibition mutant would be much lower in $CD44^{hi}$ T cells than in their naive ($CD44^{lo}$) counterparts. Instead, the transgene-encoded protein was expressed and inhibited NF-κB as much in purified $CD44^{hi}$ cells as in the $CD44^{lo}$ subset (Figure 2c, d), whereas AP-1 induction was unaffected by IκBα(ΔN) in either subset (Figure 2d). The basal NF-κB of primary T cells cannot be assayed without their purification, and it is conceivable that each of several purification techniques would activate NF-κB. Nonetheless, it is interesting to note that active NF-κB complexes were readily detectable in highly purified resting T cells (Figure 2d). Together with evidence of (micro)environmental production of TNF-α and the constitutive presence of ligands for members of the TNF-R superfamily[35,48] the data suggest that ongoing interactions with environmental stimuli lead to a low-level activity of the NF-κB pathway,[49] which is blocked by the dominant IκB transgene.

Antigen receptor signaling appears essential for the survival of naive but not memory T cells.[12–14,21] Thus, our observations could arise if TCR expression or early signaling were inhibited by the IκBα(ΔN) transgene. Importantly, mature T cells expressing IκBα(ΔN) showed normal TCR expression (both endogenous and transgenic), ZAP-70 phosphorylation, and upregulation of the early activation marker CD69 as compared to WT controls (data not shown). In addition, the expression of coreceptors and MHC proteins were all normal in IκBα(ΔN) transgenic mice (data not shown). These results indicate that the expansion of naïve-phenotype T cells is more dependent on NF-κB signaling than that of memory-phenotype cells, and suggest that the diminished expansion of IκBα(ΔN) T cells is not a consequence of reduced efficiency of TCR signaling in these mature T cell subsets.

Fundamental mechanisms which could underlie the dependence of T cell multiplication on NF-κB could include the intrinsic ability of T cells to traverse the cell cycle and the probability of T cell death. Our *in vitro* studies indicated that the inhibition of NF-κB by unknown mechanism(s) led to increased apoptotic susceptibility of mature T cells after mitogenic stimulation.[18] As high rates of ongoing apoptosis normally are hard to detect *in vivo*, even after stimulation of T

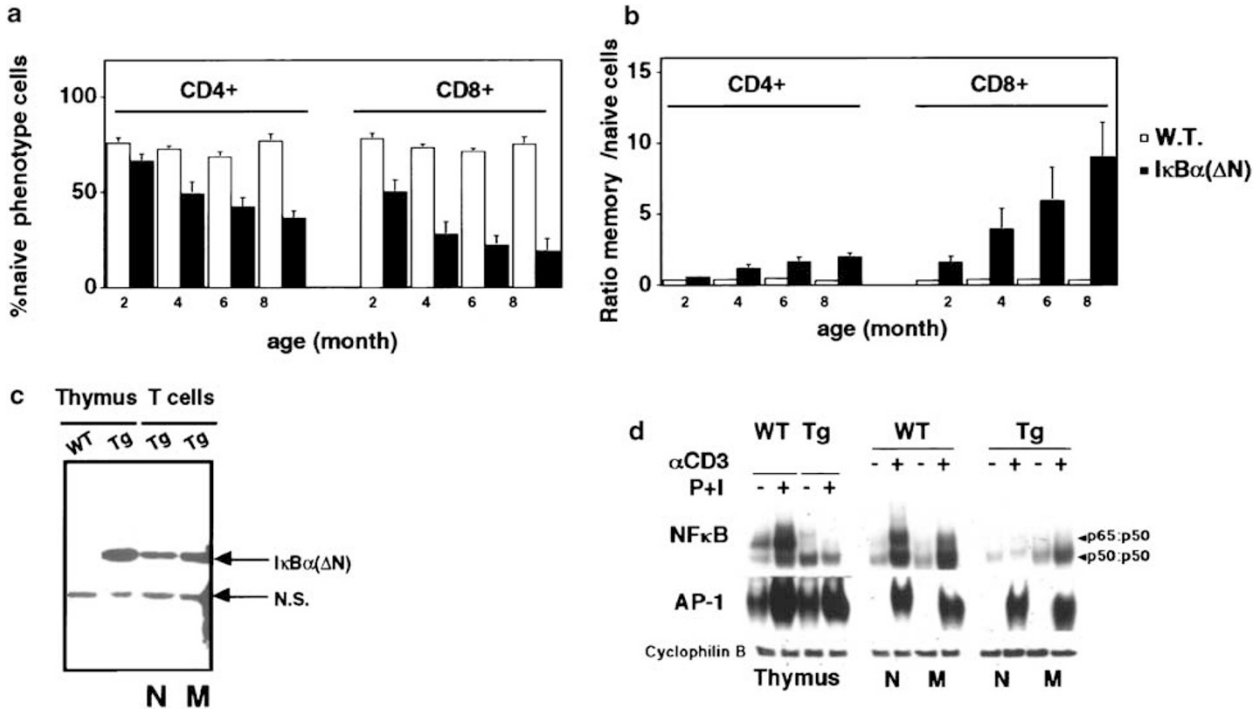

Figure 2 Sustained transgene expression and premature dominance of CD44^{hi} T cells in IκBα(ΔN) transgenic mice. (**a**,**b**) Progressive decline in naive T cells and premature dominance of CD44^{hi} T cells as a consequence of inhibiting NF-κB. Splenocytes from WT and IκBα(ΔN) transgenic littermates of the indicated ages were stained for CD4, CD8, and CD44 and analyzed by FACS. Results are presented as the mean (±S.E.M.) prevalence of CD8$^+$ or CD4$^+$ cells expressing low levels of CD44 (n=6–12 mice for each group) (panel a) as well as the mean (±S.E.M.) ratio of CD44^{hi} to CD44^{lo} cells in each mouse (panel b). The absolute numbers of CD44^{hi} cells in IκBα(ΔN) mice were roughly normal (50–90% those in WT littermates, data not shown). (**c**) Lymphocytes from WT and IκBα(ΔN) mice were stained for surface expression of CD3 and CD44 and then subjected to preparative sorting to purify CD44^{hi} and CD44^{lo} T cells. Whole cell extracts (15 μg) were subjected to anti-FLAG immunoprecipitation followed by SDS-PAGE and immunoblotting using antisera against IκBα.to determine IκBα(ΔN) expression. Thymocyte whole cell extracts (200 μg) from WT and IκBα(ΔN) mice were used as controls. (**d**) Equivalent inhibition of NF-κB induction in CD44^{hi} and CD44^{lo} T cells. Gel mobility shift analyses were performed using extracts from the indicated cell populations after preparative sorting and culture in medium supplemented as indicated (PMA/ionomycin (2 h) for thymocytes (P+I) as a control, or plate-bound anti-CD3 (16 h) for T cell subsets). Previous analyses have shown that the upper band represents p50-RelA and c-Rel containing complexes, whereas the lower band corresponds primarily to complexes lacking RelA and c-Rel. As controls, matched amounts of the same extracts were used for mobility shift assays using radiolabeled AP-1 probe and in immunoblots of cyclophilin B, as indicated

cells, we measured the death rate of 1B2$^+$ CD8$^+$ cells from 2C TCR-transgenic mice 2 days after adoptive transfer into recently irradiated BALB/c recipients. The proportion of dead 1B2$^+$ CD8$^+$ cells was two-fold higher among donor cells from IκBα(ΔN) mice as compared to WT donors (Figure 3a). To investigate the role of NF-κB in regulating apoptotic susceptibility of mature T cells by a transgene-independent approach, we used retroviral transduction of developmentally normal T cells. When the IκBα(ΔN) protein was expressed in recently activated primary T cells, which were then re-stimulated, a significantly higher rate of restimulation-induced apoptosis was observed in both CD4$^+$ and CD8$^+$ T cells whose NF-κB induction was inhibited (Figure 3b). Together, these findings indicate that one mechanism by which NF-κB regulates peripheral T cell numbers is by promoting protection against cell death.

NF-κB protects cells from Fas *in vivo*

Engagement of the death receptor Fas by FasL plays an important role in regulating the populations of CD4 and CD8 T lymphocytes by inducing cell death. To determine if NF-κB promotes survival of T cells *in vivo* by providing protection against normal processes of Fas-mediated apoptosis, we crossed the IκBα(ΔN) transgene onto a background that harbors a loss-of-function mutation in the fas gene. The loss of Fas completely reversed the defect in cellularity of the CD4$^+$ T cell subset caused by expression of IκBα(ΔN) in these cells (Figure 4a). The numbers of CD8$^+$ T cells doubled (Figure 4b), consistent with prior work showing Fas-dependent death of CD8 T cells *in vitro* and *in vivo*.[50–52] In contrast to the results with CD4-lineage T cells, however, this increase represented only a partial recovery of the population size. This distinction suggests that the molecular requirements for NF-κB in providing protection against Fas-dependent apoptosis *in vivo* depend on the differentiated state of the T lymphocyte, so that CD4 lineage cell numbers were completely restored whereas CD8 cells were not.

The increased size of peripheral T cell populations observed in these epistasis experiments *in vivo* was associated with a decrease in activation-induced cell death of CD4$^+$ and CD8$^+$ IκBα(ΔN) T cells bearing the lpr mutation (Figure 4c). Apoptotic responses to Fas and other death receptors are controlled by a set of inducible inhibitors. Among these, apoptosis inhibitors termed c-FLIP (FLICE/caspase-8 inhibitory proteins) inhibit the activation of caspase-8 at the level of a death-inducing signaling complex (DISC) formed by the activation of Fas. To determine if NF-κB regulates FLIP

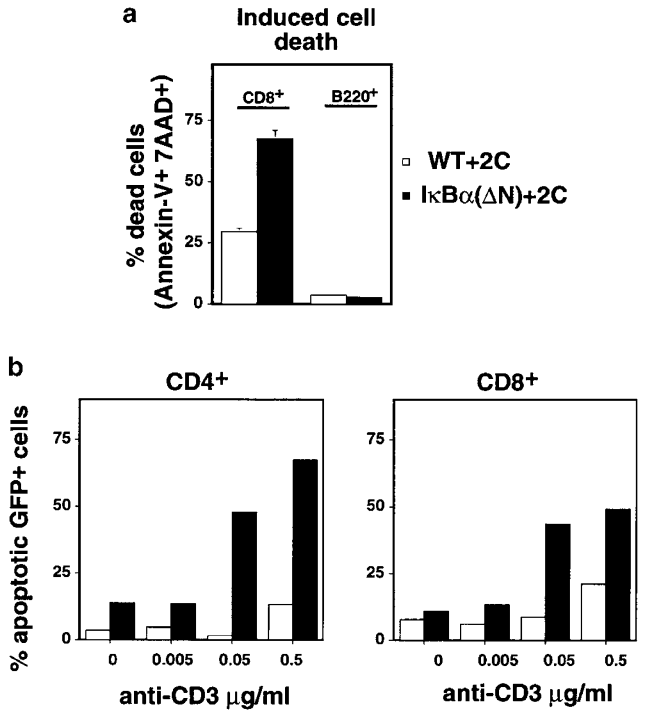

Figure 3 Increased susceptibility of IκBα(ΔN) T cells to death *in vivo*. (**a**) Lymphoid cells from H-2^b, 2C TCR transgenic mice bearing or lacking the IκBα(ΔN) transgene were transferred into irradiated BALB/c recipients (10^6 1B2$^+$ CD8$^+$ cells/mouse) as described in Figure 1b. Induced cell death was determined by Annexin-V/7AAD staining 2 days after adoptive transfer. Representative data are presented as the mean percentage of dead cells among CD8$^+$ or B220$^+$ cells. (**b**) NF-κB regulates susceptibility to AICD in developmentally normal T cells. Lymphoid cells from B6 mice were activated with ConA (2.5 μg/ml). After 24 h, cells were transduced with an IRES-GFP retrovirus containing no additional insert or the IκBα(ΔN) cDNA and maintained 48 h in the presence of IL-2. After 2 days, cells were restimulated with the indicated concentrations of plate-bound anti-CD3 mAb. Cell viability was determined by FACS analysis measuring the percentage of 7-AAD positive cells from CD4$^+$ and CD8$^+$ gate

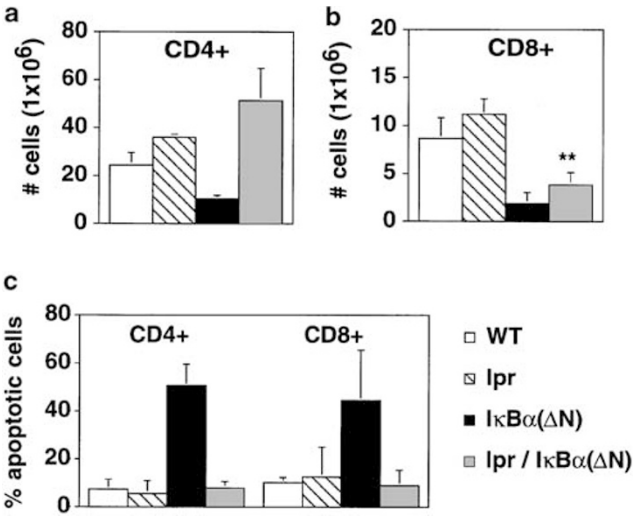

Figure 4 NF-κB regulates T cell numbers through a Fas-dependent mechanism. Lack of Fas ameliorates the population defect of T cells in IκBα(ΔN) mice. B6 lpr/lpr and IκBα(ΔN) transgenic mice were intercrossed, and lymphoid cells from young WT, lpr/lpr, IκBα(ΔN) and lpr/lpr, IκBα(ΔN) littermate mice were counted, stained for surface expression of CD4 and CD8, and analyzed by FACS. The data represent the mean numbers of total T cells (±S.E.M.) (number of cells in spleen+2.5 × number of cells in lymph nodes,[4]) for the CD4$^+$ (A) or CD8$^+$ (B) subset ($n \geq 4$ mice for each group) (**, $P < 0.01$ *versus* IκBα(ΔN) sample set). (**c**) Splenocytes from mice of the indicated genotype were cultured 48 h following ConA stimulation (2.5 μg/ml) and then restimulated at a limiting concentration of anti-CD3 (50 ng/ml). Cells were harvested, surface stained for CD4 and CD8 expression and subjected to TUNEL analysis. Data are presented as the mean of the percentage of TUNEL-positive cells in the CD4$^+$ or CD8$^+$ gates. Results represent the mean values (±S.E.M.) from two separate experiments, two mice in each group

expression in primary T cells, samples from WT and IκBα(ΔN) transgenic mice were activated and analyzed by Western blotting. Naive IκBα(ΔN) T cells expressed significantly lower levels of c-FLIP following stimulation (Figure 5). Together, these results indicate that one of the mechanisms by which NF-κB participates in regulating T cell population size *in vivo* is by providing protection against Fas-mediated death, and suggest that a role of NF-κB in promoting c-FLIP expression helps to mediate this resistance to Fas.

An NF-κB-dependent gene product, Bcl-x$_L$, suppresses effects of IκBα(ΔN) *in vivo*

Although a significant increase in the CD8 subset was observed in Fas-deficient CD8 T cells subject to inhibition of the NF-κB, CD8 T cell numbers in the IκBα(ΔN) transgenic mice lacking functional Fas were substantially diminished. This finding suggested that NF-κB might also regulate other apoptotic signaling pathways in primary mouse T cells. Antiapoptotic members of the Bcl-2 gene family can inhibit the cell death promoted by Fas-independent mechanisms, and screens of defects in Jurkat T cells expressing IκBα(ΔN) revealed decreased induction of Bcl-x$_L$ but not Bcl-2 (data not

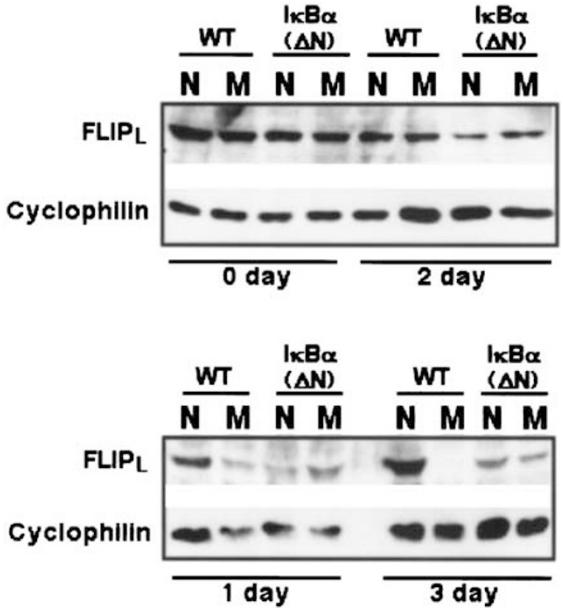

Figure 5 NF-κB regulation of c-FLIP levels. T cell-enriched preparations from WT and IκBα(ΔN) mice were prepared by negative selection of MHC class II$^+$ cells followed by separation of CD62L^{hi} (naive – N) and CD62L^{lo} (memory-phenotype – M) T cells. After stimulation for the indicated time using plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (2.5 μg/ml), whole cell extracts were subjected to SDS-PAGE and resolved proteins (6 μg in each lane) were detected by Western blotting for c-FLIP. Relative loading was assessed by reprobing blots with anti-cyclophilin B

shown). Moreover, in contrast to findings reported for immature thymocytes subjected to inhibition of NF-κB,[28] Bcl-x_L mRNA and protein expression in T cells from IκBα(ΔN) mice were attenuated compared to WT controls (Figure 6a, b). Of note, analyses of RNA from freshly isolated splenic T cells confirmed prior evidence that Bcl-x mRNA is expressed in resting T cells.[53] Together with the results of transient transfection assays showing NF-κB-dependent *trans*-activation of a Bcl-x promoter segment in T cells (data not shown), the data indicate that Bcl-x_L gene expression is directly mediated by NF-κB/Rel transcription factors in normal $CD4^+$ and $CD8^+$ T lymphocytes.

To test *in vivo* the functional significance of this dependence, an epistasis analysis was performed by mating IκBα(ΔN) transgenic mice with a transgenic line that targets constitutive expression of Bcl-x_L to the T lineage. This transgene leads to Bcl-x_L expression in both thymocytes and mature T cells, and protected a significant fraction of mature $CD4^+$ and $CD8^+$ T cells from apoptosis.[27] The level of transgenic Bcl-x_L expression in mature $CD4^+$ and $CD8^+$ T cells was equal to that attained by WT cells after TCR stimulation [27] (data not shown). Restoration of Bcl-x_L expression to the physiological level of activated T cells partially suppressed the

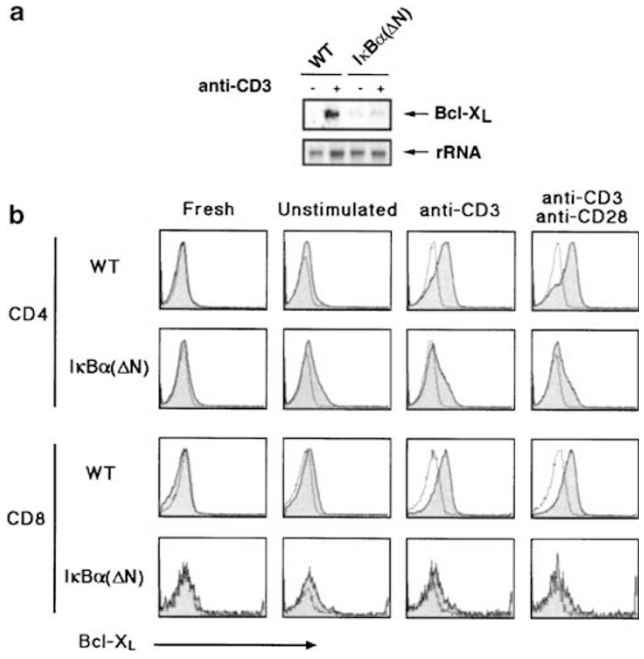

Figure 6 Bcl-x_L induction depends on NF-κB induction in primary T cells. (**a**) T cell-enriched preparations from pooled spleen and LN of WT and IκBα(ΔN) transgenic mice were cultured overnight, alone or with plate-bound anti-CD3 (10 μg/ml). Total RNA preparations (5 μg per sample) were fractionated on denaturing gels, transferred to nylon membranes and probed with a radiolabeled Bcl-x cDNA. (**b**) NF-κB-dependent induction of Bcl-x_L protein in T cells. Bcl-x_L expression was measured by intracellular staining of Bcl-x_L expression in $CD4^+$ and $CD8^+$ splenocytes (freshly isolated or cultured 16 h in medium, alone or with plate-bound anti-CD3 (10 μg/ml) or anti-CD3 plus anti-CD28 (2.5 μg/ml), as indicated). Owing to the paucity of T lymphocytes (especially $CD8^+$ cells) in IκBα(ΔN) samples, the number of events shown in each panel has been adjusted to equalize numbers among the CD4-gated samples and similarly among CD8-gated samples; in the unadjusted samples, the absolute frequencies of $CD4^+$ and $CD8^+$ cells correspond to data in Figure 4. The effect of IκBα(ΔN) on Bcl-x_L induction was similar in naive as compared to memory-phenotype $CD4^+$ and $CD8^+$ cells (not shown)

homeostatic defect created by NF-κB blockade *in vivo*, leading to increased steady-state populations of T cells. Within lymph nodes, a preferred site for naive cells, and spleen, $CD4^+$ cell numbers were restored to normal levels (Figure 7a; data not shown). In contrast, there was little increase because of Bcl-x_L expression in single transgenics. Similar to the findings with Fas-deficient IκBα(ΔN) T cells, $CD8^+$ cell numbers increased significantly in doubly transgenic (IκBα(ΔN) × Bcl-x_L) mice but this increase was insufficient to restore a normal population size. These results represented specific epistatic suppression rather than a nonspecific effect of Bcl-x in increasing cell numbers (Figure 7b).

The lack of naive T lymphocytes and relatively modest diminution in numbers of memory-phenotype cells (Figure 2) suggested that net losses from an increased death rate impact naive T cells more than the memory-phenotype subset and that a deficit of antiapoptotic proteins in the Bcl-2 superfamily contributed to this imbalance. Importantly, constitutive expression of Bcl-x_L restored a largely normal ratio of naive ($CD44^{lo}$) to memory-phenotype ($CD44^{hi}$) cells in IκBα(ΔN) transgenic mice (Figure 7c). Caspase-3 activation can be a critical step in cellular commitment to apoptosis, and epistatic analysis indicates that it is a key step downstream from Fas.[54] Taken together with results on cell numbers, this finding indicates that Bcl-x_L had a greater effect on the naive population when NF-κB was inhibited in $CD4^+$ and $CD8^+$ T lymphoid lineages. Measurement of activated caspase-3 in CD4 and CD8 T cells analyzed immediately *ex vivo* showed that for both of these lineages, caspase-3 activation was increased in IκBα(ΔN) T cells as compared to WT controls. This finding reinforces the conclusion from adoptive transfers of CD8 T cells (Figure 3a), to the effect that enhanced rates of apoptosis *in vivo* result from inhibition of NF-κB in mature T cells. Importantly, enforced expression of Bcl-x_L in IκBα(ΔN) T cells suppressed the increase in activated caspase-3 (Figure 7d). Together, the findings indicate that NF-κB/Rel proteins regulate T cell numbers at least in part by controlling apoptotic susceptibility *in vivo*. However, the involvement of a nonredundant homeostatic mechanism, especially in the CD8 lineage of T cells, is suggested by the finding that neither enforced Bcl-x_L nor the absence of Fas receptor completely restored this population to normal when NF-κB was inhibited *in vivo*.

NF-κB blockade causes apoptosis-independent perturbations of cell cycling *in vitro*

Prior work has shown that T lymphocytes from IκBα(ΔN) transgenic mice exhibit a drastic decrease in cytokine induction of Stat5, which is crucial for T cell proliferation,[55,56] and NF-κB regulates cyclin D1 in nonlymphoid cell lines.[57,58] However, Stat5 may provide a survival signal,[59] and there is little if any cyclin D1 expression in T cells. For kinetic reasons, labeling techniques *in vivo* are unable to provide a definitive measurement of division rates in the face of the substantial enhancement of apoptosis, so *ex vivo* studies were performed. To distinguish an effect on cell cycling from the effects of IκBα(ΔN) on T cell survival prior to a [3-H]-TdR pulse, we quantitated proliferation of mitogenically activated $CD4^+$ and

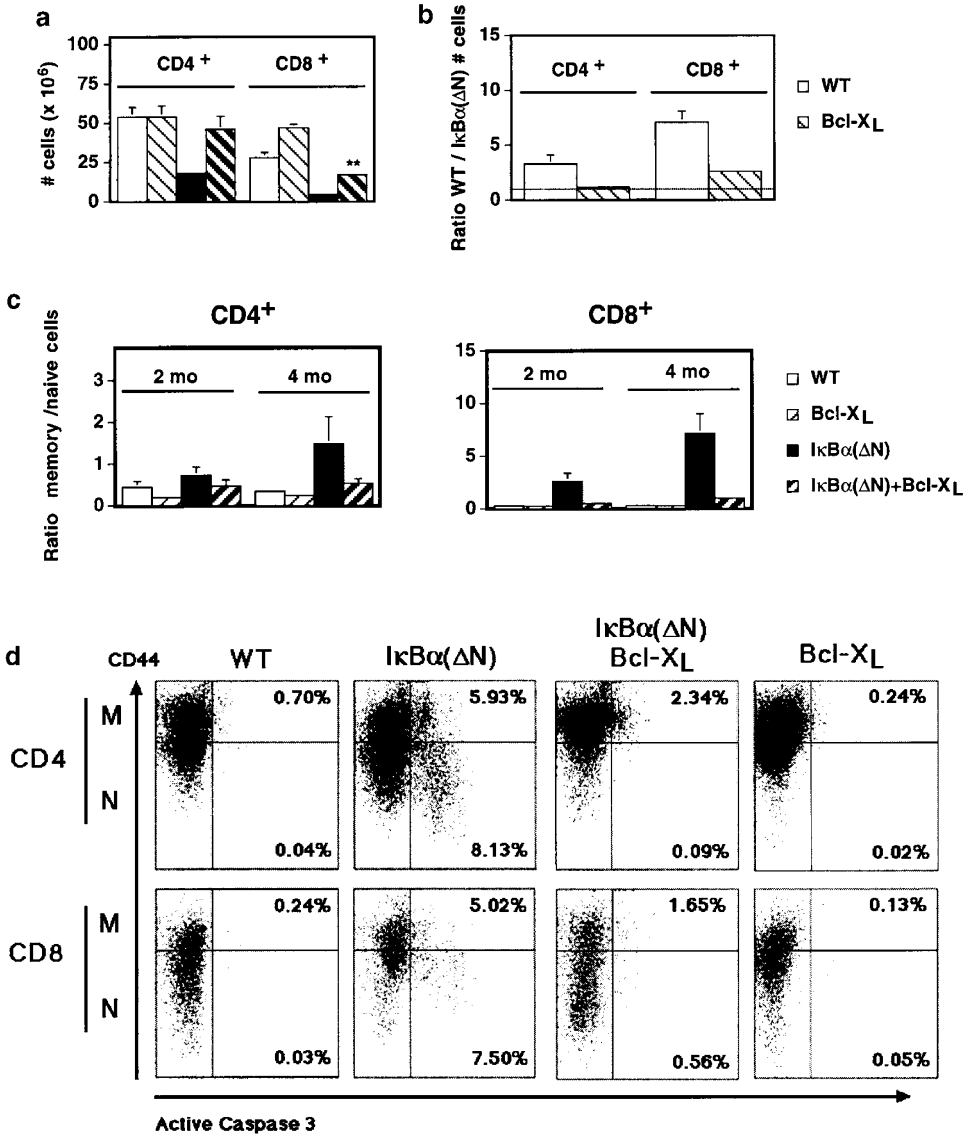

Figure 7 Bcl-x_L and homeostatic regulation by NF-κB.Bcl-x_L ameliorates the T cell population defect caused by inhibition of NF-κB/Rel signaling. (**a**,**b**) Cells from lymph nodes of the indicated mice were counted, stained for CD4 and CD8, and analyzed by FACS. The data represent the mean numbers (±S.E.M.) of $CD4^+$ and $CD8^+$ cells ($n \geq 6$ mice for each group) (panel b) as well as the mean (±S.E.M.) ratio of number of cells from wild type/IκBα(ΔN) mice matched for the presence of the Bcl-x_L transgene (WT/IκBα(ΔN), open bars; Bcl-x_L/IκBα(ΔN), Bcl-x_L double Tg, hatched bars) (panel b). (**c**) Bcl-x_L normalizes the imbalance of naive- and memory-phenotype cells imposed by IκBα(ΔN). Splenocytes from 2- and 4-month old littermates of the indicated genotypes were stained for surface expression of CD4, CD8, and CD44, then analyzed by FACS ($n \geq 6$ for each age and genotype). Shown are the means (±S.E.M.) of the ratio of $CD44^{hi}$ to $CD44^{lo}$ cells for $CD4^+$ and $CD8^+$ cells in each mouse. (**d**) Bcl-x_L suppresses increases in activated caspase-3 caused by inhibition of NF-κB in T cells *in vivo*. Splenocytes from unmanipulated mice bearing the indicated transgenes were stained for CD4 or CD8, CD44, TCRβ, and caspase-3 immediately *ex vivo*. Representative profiles are shown after gating on TCRβ-positive cells in the CD4 and CD8-positive gates, as indicated. Owing to the paucity of T lymphocytes (especially $CD8^+$ cells) in IκBα(ΔN) samples, the number of events shown in each panel has been adjusted to equalize numbers among the CD4-gated samples and similarly among CD8-gated samples; in the unadjusted samples, the absolute frequencies of $CD4^+$ and $CD8^+$ cells correspond to data in panel A. A division into naive- (N) and memory- (M) phenotypes according to CD44 hyperexpression is indicated

$CD8^+$ T cells with brief BrdU pulse labeling. This analysis demonstrated a lower rate of entry into S-phase for both $CD4^+$ and $CD8^+$ T cells derived from the IκBα(ΔN) transgenic mice as compared to WT controls (Table 1). Thus, proliferation *in vitro* was decreased when measured under conditions that avoid effects because of differential rates of developmental progression or death.

To determine whether increased Bcl-x_L levels lead to increases in the multiplication of T lymphocytes subjected to NF-κB blockade, rates of ^{3}H-thymidine incorporation were measured for WT, Bcl-x_L, IκBα(ΔN), and double-transgenic cells after mitogenic stimulation (Figure 8a). Restoration of Bcl-x_L levels to those of activated T cells led to no significant increase in proliferation in a population comprising both $CD4^+$ and $CD8^+$ T cells. Further, $CD4^+$ T cell multiplication *in vivo* was measured after adoptive transfers of limited numbers of antigen-specific T cells expressing combinations of the IκBα(ΔN) and Bcl-x_L transgenes and immunization of the

Table 1 Apoptosis-independent decrease in cell cycling in vitro

	Br-dU uptake	
	WT	**IκBα(ΔN)**
$CD4^+$	11.7 (3.3)[a]	5.8 (1.7)
$CD8^+$	17.2 (4.4)	9.3 (1.2)

Splenocytes from WT and IκBα(ΔN) mice activated by T-cell mitogen were cultured 1 h in the presence of bromodeoxyuridine (BrdU) 48 h after stimulation. Pulsed cells were stained for CD4, CD8, and BrdU, and analyzed by flow cytometry. [a]Mean (±S.E.M.) percentages of BrdU-positive cells in the indicated cellular subsets.

recipients with antigenic peptide (Figure 8b). These experiments showed that the Bcl-x_L transgene did not overcome the block to clonal expansion caused by inhibition of NF-κB in CD4 T cells. These combinations of the IκBα(ΔN) and Bcl-x_L transgenes were also used in experiments measuring the efficiency of blast formation, ^{3}H-uridine incorporation (G0, G1 stages of cycling), and BrdU incorporation during short pulses (S-phase) (Figure 8c-e). Each of these parameters of cell cycle kinetics was decreased in $CD4^+$ T cells expressing IκBα(ΔN), and constitutive Bcl-x_L expression failed to reverse any of the defects caused by inhibition of NF-κB (Figure 8c-e). Together, these data indicate that although normal numbers of $CD4^+$ T cells were restored by the enforced expression of Bcl-x_L, the effect was not accompanied by a substantial increase in their cell cycling efficiency i*n vitro* or activation-induced multiplication *in vivo*.

Discussion

NF-κB/Rel transcription factors participate in a range of developmental processes including the regulation of cell death. Depending on the cell type, particular subunits of this multigene family either promote[60,61] or protect against apoptosis[62] in response to stimuli, and can apparently serve opposite roles within the same cell type depending on the stimulus.[61,63] We have used the T lymphoid lineage as a model system to explore the relation between differentiation status and NF-κB-dependent regulation of cell population size and apoptosis. No individual knockout of a gene encoding NF-κB/Rel subunits has been shown to play a cell-intrinsic role in regulating the population size of mature lymphoid cells (reviewed in Ghosh *et al*.[15]). Using T cell-specific expression of a transgene to inhibit NF-κB, we provide evidence that NF-κB regulates homeostasis of mature T cells through regulation of their rates of apoptosis. Strikingly, however, the data reveal that the impact of NF-κB and a downstream anti-apoptotic gene product, Bcl-xL, on survival and population size depend on the state of differentiation within the T lineage.

Specifically, we show that NF-κB promotes the survival of mature T cells *in vivo*, particularly those of naive phenotype. This preferential effect of the inhibitory transgene on naive cells parallels the finding that blockade of NF-κB disproportionately slowed population expansion of naive T cells in the intact mouse, as compared to their memory-phenotype descendants. Further, the evidence suggests that there are at least two distinct mechanisms by which these transcription

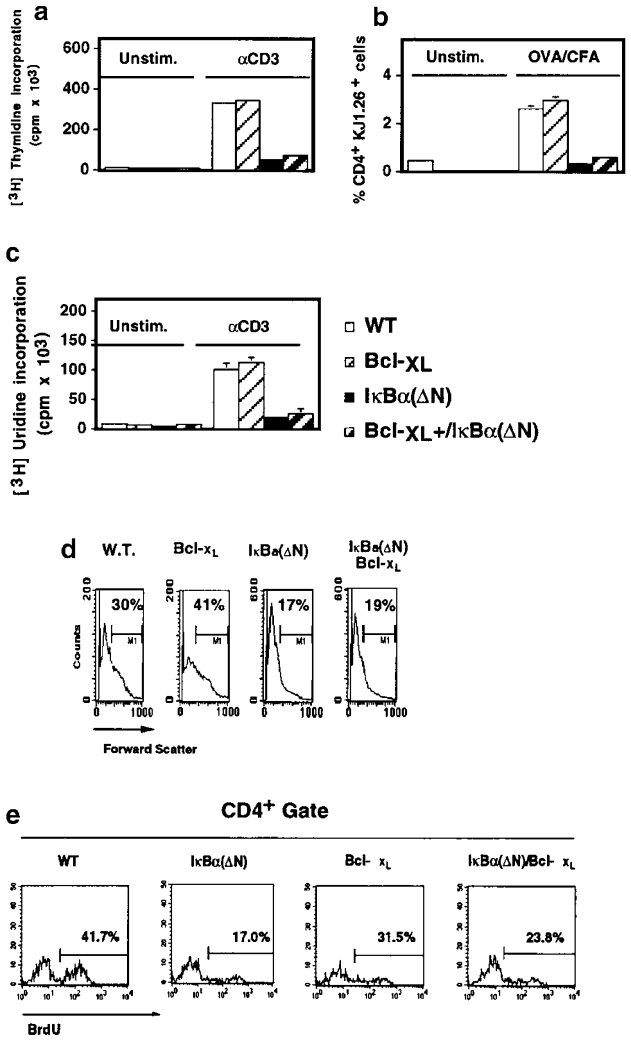

Figure 8 Failure of Bcl-x_L to reverse defective proliferation of IκBα(ΔN) T cells. (**a**) Splenocytes from mice of the indicated phenotypes were cultured for 40 h with medium alone or plate-bound anti-CD3 (10 μg/ml), pulsed for 8 h with ^{3}H-TdR, and counted. Data represent mean values in one experiment of four performed with two mice for each group. (**b**) Lymph node cells from BALB/c DO-11.10 TCR transgenic mice expressing Bcl-x_L, IκBα(ΔN) and Bcl-x_L, IκBα(ΔN) double-transgenic littermates were transferred into syngeneic recipients. These mice were immunized with OVA peptide in CFA, and 7 days later cells harvested from draining lymph nodes were analyzed by FACS after staining with CD4 and the clonotypic antibody for the TCR (KJ1-26). Data are presented as the mean percentages of $CD4^+$ $KJ1\text{-}26^+$ cells (±S.E.M.) in one experiment of the four performed and represent four to eight mice for each group. (**c**) Splenocytes from mice of the indicated phenotypes were activated as in (**a**) and pulsed (8 h) with tritiated uridine as in Grumont *et al*.[31] Data represent one experiment of the three performed (n=2 mice for each group). (**d**) Splenocytes from littermates of the indicated phenotype were cultured for 40 h with plate-bound anti-CD3 (10 μg/ml) followed by FACS analysis. Representative forward scatter histograms from the $CD4^+$ gate are shown. Similar results were obtained for $CD8^+$ cells. Numbers represent the percentage of cells with high forward scatter (blasts). (**e**) Lymphocyte cell preparations from DO-11.10 TCR transgenic mice expressing Bcl-x_L, IκBα(ΔN) and Bcl-x_L, IκBα (ΔN) double-transgenic littermates were treated for 40 h with plate-bound anti-CD3 (10 μg/ml) and then pulsed with BrdU (2 h). BrdU incorporation was measured by FACS analysis gated on $CD4^+$ $KJ1.26^+$ cells. The percentage of $BrdU^+$ T cells is indicated in the representative histograms

factors influence the maintenance of normal T cell numbers. First, NF-κB regulates Bcl-x_L induction in these primary cells and promotes T lymphocyte survival *in vivo*, with epistasis analyses indicating that a Bcl-x_L transgene ameliorates abnormalities of T cell number and decreases the frequency of T cells with activated caspase-3 in the IκBα(ΔN) background. However, Bcl-x_L-mediated increases in cell numbers most impacted naive and $CD4^+$ cells, such that inhibition of apoptosis normalized the ratio between naive and memory-phenotype T cells. Second, NF-κB also plays a role in the protection of naive T cells against the death receptor Fas. When NF-κB signaling was impaired in Fas-deficient T cells *in vivo*, the deficit of $CD4^+$ lymphocyte numbers was completely reversed, and $CD8^+$ T cell numbers increased but were not restored to normal These findings are consistent with prior evidence of a significant role for Fas in regulating naive T cells.[51]

Our epistasis results indicate that NF-κB mediates protection against apoptosis promoted through the Fas death receptor in primary T cells *in vivo*. This effect was correlated with a role for NF-κB in determining the steady-state level of the survival factor c-FLIP. These findings are consistent with a model in which NF-κB regulates Fas-mediated cell death via levels of c-FLIP. NF-κB-dependent induction of c-FLIP has been observed in overexpression systems using reporter plasmids,[64] and FLIP levels are associated with protection against Fas-mediated AICD in primary T cells.[65,66] A uniform role of Bcl-x_L in preventing Fas-inducing apoptosis has been elusive, but our aggregate data (Figures 6, 7 suggest that NF-κB-dependent Bcl-x_L induction contributes to regulating survival of naive $CD4^+$ T cells after FasL encounters *in vivo*. Expression of Bcl-x_L may impede Fas-mediated cell death in some settings, but the Fas/FasL pathway can induce death by separable pathways, only one of which is blocked by prosurvival members of the Bcl-2 superfamily.[67,68] Our results suggest that the NF-κB dependence of activation-induced Bcl-x_L expression in primary T cells helps to control their fate, especially for $CD4^+$ cells. Thus, expression of this survival factor in mature lymphoid cells at the level induced by T cell activation (data not shown) leads to a specific but partial suppression of the deficit of naive T cells and the imbalance between naive- and memory-phenotype T lymphocytes (Figure 7).

We have found that normal numbers of $CD4^+$ T cell are restored either by the absence of Fas or by enforced Bcl-x_L expression when NF-κB is inhibited *in vivo*. These data suggest that these are key mechanisms through which NF-κB acts in this lineage. The significant increases in $CD8^+$ T cells implicate these molecular links in regulation of CD8 T cell numbers. However, the failure of either Fas deficiency or constitutive Bcl-x_L expression to restore normal numbers of $CD8^+$ T cells indicates that additional mechanisms are involved in the regulation of homeostasis by NF-κB. Two major possibilities, which are not mutually exclusive, are that (i) the cell cycling defects observed *in vitro* are applicable *in vivo*, or (ii) Fas-independent apoptotic pathways that require survival factor(s) other than Bcl-x_L are responsible. Roles for NF-κB/Rel proteins in regulating survival pathways in other cell types have been proposed, but the relation of most to normal T cells is unclear. Analyses of Jurkat T cells indicated that induction of mRNA encoding the Bcl-2/X-related protein Bfl-1/A1 was also blocked by IκBα(ΔN) (data not shown). Antiapoptotic Bcl-2-related proteins substitute functionally for one another in transgenic and knockout mice,[27] so the Bcl-x_L transgene used in the present study may have reversed a decrease in Bfl-1 in addition to that noted for Bcl-x_L. Outside the Bcl-2 family, several other molecules with prosurvival function have been reported to be NF-κB target genes in various tumor cell lines. Normal levels and induction of mRNAs encoding c-myc,[55] caspase inhibitors (cIAP-1, cIAP-2), and the TNF-receptor associated factors TRAF1 and TRAF2 were found in IκBα(ΔN) T cells (data not shown). Nonetheless, death receptors other than Fas and defects in still other NF-κB-dependent survival proteins such as GADD45β[69] or Bcl-3 are also likely to be implicated in the enhanced death of IκBα(ΔN)-expressing T cells.

Intriguingly, NF-κB was found to regulate the kinetics of $CD4^+$ and $CD8^+$ T cell cycling in short-pulse BrdU labeling experiments *in vitro*. Whether this finding applies to mature T cells *in vivo* remains unclear. A 50% decrease in the numbers of stage III $CD4^-CD8^-TCR^-$ thymic precursors to T cells was attributed exclusively to enhanced susceptibility to apoptosis.[36] However, the quantitatively greater severity of the population defect for mature T cells in IκBα(ΔN) mice might reflect a dual role, with NF-κB promoting cell cycle rates in addition to the effect on survival. The *in vitro* data indicate that inhibition of NF-κB in T cells can lead to intrinsic defects in execution of steps in cell cycle progression, at least outside of the normal microenvironment. Previous studies in other cell types suggest that NF-κB may participate in the control of cell cycle regulatory proteins[57,58,70] but the role of NF-κB in cell proliferation appears dependent on the tissue and cell type. RNA synthesis and blastogenesis of $CD4^+$ IκBα(ΔN) transgenic T cells were substantially decreased compared to controls, effects which persisted despite constitutive expression of Bcl-x_L (Figure 8). Although the analysis with Bcl-x_L transgenic cells was limited to CD4-lineage T cells for technical reasons, the lack of any meaningful increase in 3-H TdR incorporation into a mixed population of cycling CD4 and CD8 T cells suggests that enforced Bcl-x_L expression also was unable to enhance the proliferation of the CD8 subset. The BrdU pulse labeling studies suggest that NF-κB regulates the rate of entry into S phase (Table 1; Figure 8). In a mammary tumor cell line, NF-κB activity has been associated with cyclin D1 transcription in the G1 phase and the G1-to-S-phase transition,[58] whereas NF-κB appears to control p53, p21WAF, and c-Myc in HeLa cells.[70] We have recently determined that c-Myc induction is unaffected by the inhibition of NF-κB in primary T cells.[55] However, cytokine-activated Stat5 is essential for cell cycle progression in T cells and the T cell-specific expression of IκBα(ΔN) selectively inhibits Stat5 induction by cytokine receptors which share the γc chain (IL-2R and IL-4R).[55,59,71] Although its role *in vivo* remains unproven, this defect in cytokine-induced Stat5 could potentially contribute to both the apoptosis-independent cycling defects *in vitro* and to defects in survival.

Unlike memory T cells, the survival of naive lymphocytes requires ligand engagement of the antigen receptor.[12–14] This observation raises the question whether the NF-κB-dependence of T cell survival and clonal expansion exclusively

reflect TCR signaling. PKC-θ is an essential link between TCR signaling and NF-κB activation in mature T lymphocytes.[72,73] T cells derived from PKC-θ -deficient mice exhibited impaired activation, proliferative responses (*in vitro*), and a defect in T cell-dependent function (*in vivo*), yet T cell numbers were normal in PKC-θ knockout mice.[72] These results suggest that decreased survival and a modest decrease in the initial rate of T cell production arise when NF-κB induction by a wide variety of signaling pathways is inhibited (as in IκBα(ΔN) mice) as opposed to a selective defect in induction by the antigen receptor. Thus, it is likely that NF-κB activation by PKC-θ -independent pathways, such as those triggered by members of the TNF and IL-1-receptor superfamilies, are critical for the generation of NF-κB-dependent survival signals in T cells in their microenvironment.

Acknowledgements

We gratefully acknowledge expert technical assistance from W Armistead, B Enerson, S McCarthy, and M McReynolds. We thank P Fink, R Merica, T Hettman, Y Choi, G Oltz, J Chen, and A Chan for helpful discussions; J Chen, G Oltz, Y Choi, G Miller, and D Unutmaz, for critical readings of manuscript drafts; R Merica and M Jenkins, H-S Teh, AH Lichtman, and G Cheng for generous gifts of reagents and mouse lines; Immunex Corp and FW Alt for cDNAs; J Price and D McFarland for expert flow cytometry through the Vanderbilt Cancer Center and HHMI FACS cores, and the Vanderbilt Ingram Cancer Center (NIH grant CA68485) and Diabetes Research and Training Center (P60 DK20593) for Tissue Culture, DNA, Molecular Biology and Flow Cytometry core functions and a Pilot Project (the Mark Collie Fund and DK20593). RAC was supported by an NIH training grant to the Meharry-Vanderbilt Alliance (R25 GM62459), MB was a Scholar of the Leukemia Society of America, and funding for this work was provided to MB and SJ by the NIH (AI-36997; HL-61752; AI-49460); AI42284), JDRF (SJ), and Vanderbilt Discovery Pilot program (MB).

References

1. Berzins SP, Boyd RL and Miller JF (1998) The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J. Exp. Med. 187: 1839–1848
2. Berzins SP, Godfrey DI, Miller JF and Boyd RL (1999) A central role for thymic emigrants in peripheral T cell homeostasis. Proc. Natl. Acad. Sci. USA 96: 9787–9791
3. Freitas AA and Rocha B (1999) Peripheral T cell survival. Curr. Opin. Immunol. 11: 152–156
4. Rocha B, Dautigny N and Pereira P (1989) Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. Eur. J. Immunol. 19: 905–911.
5. Tanchot C, Rosado MM, Agenes F, Freitas AA and Rocha B (1997) Lymphocyte homeostasis. Semin. Immunol. 9: 331–337
6. Sprent J, Schaefer M, Hurd M, Surh CD and Ron Y (1991) Mature murine B and T cells transferred to SCID mice can survive indefinitely and many maintain a virgin phenotype. J. Exp. Med. 174: 717–728
7. Bender J, Mitchell T, Kappler J and Marrack P (1999) CD4+ T cell division in irradiated mice requires peptides distinct from those responsible for thymic selection. J. Exp. Med. 190: 367–374
8. Goldrath AW and Bevan MJ (1999) Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. Immunity 11: 183–190
9. Goldrath AW and Bevan MJ (1999) Selecting and maintaining a diverse T cell repertoire. Nature 402: 255–262
10. Laouar Y and Crispe IN (2000) Functional flexibility in T cells: independent regulation of CD4+ T cell proliferation and effector function *in vivo*. Immunity 13: 291
11. Dutton RW, Bradley LM and Swain SL (1998) T cell memory. Annu. Rev. Immunol. 16: 201–223.
12. Ernst B, Lee DS, Chang JM, Sprent J and Surh CD (1999) The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity 11: 173–181
13. Tanchot C, Lemonnier F, Perarnau B, Freitas A and Rocha B (1997) Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science 276: 2057–2062
14. Viret C, Wong FS and Janeway CA (1999) Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. Immunity 10: 559–568
15. Ghosh S, May MJ and Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16: 225–260
16. Jamieson C, McCaffrey PG, Rao A and Sen R (1991) Physiologic activation of T cells via the T cell receptor induces NF-kappa B. J. Immunol. 147: 416–420
17. Sen J, Venkataraman L, Shinkai Y, Pierce JW, Alt FW, Burakoff SJ and Sen R (1995) Expression and induction of nuclear factor-kappa B-related proteins in thymocytes. J. Immunol. 154: 3213–3221
18. Boothby MR, Mora AL, Scherer DC, Brockman JA and Ballard DW (1997) Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of nuclear factor (NF)-kappaB. J. Exp. Med. 185: 1897–1907
19. Mora AL, Chen D, Boothby M and Rubin DH (1999) Lineage-specific differences among CD8+ T cells in their dependence of NF-kappa B/Rel signaling. Eur. J. Immunol. 29: 2968–2980
20. Hettmann T and Leiden JM (2000) NF-kappa B is required for the positive selection of CD8+ thymocytes. J. Immunol. 165: 5004–5010
21. Boothby M, Mora AL and Stephenson L (2002) Lymphokine-dependent proliferation of T lymphoid cells: regulated responsiveness and role in vivo. Critical Rev. Immunol. 21:487–522
22. Schluns KS, Kieper W, Jameson SC and Lefrancois L (2000) Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat. Immunol. 1: 426–432
23. Ku CC, Murakami M, Sakamoto A, Kappler J and Marrack P (2000) Control of homeostasis of CD8+ memory T cells by opposing cytokines. Science 288: 675–678
24. Zhang X, Sun S, Hwang I, Tough DF and Sprent J (1998) Potent and selective stimulation of memory-phenotype CD8+ T cells *in vivo* by IL-15. Immunity 8: 591–599
25. Brockman JA, Scherer D, McKinsey T, Hall S, Qi X, Lee W and Ballard DW (1995) Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. Mol. Cell. Biol. 15: 2809–2818
26. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH and Loh DY (1988) Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature 336: 73–76
27. Chao DT, Linette GP, Boise LH, White LS, Thompson CB and Korsmeyer SJ (1995) Bcl-xL and Bcl-2 repress a common pathway of cell death. J. Exp. Med. 182: 821–828
28. Hettmann T, DiDonato J, Karin M and Leiden JM (1999) An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis. J. Exp. Med. 189: 145–158
29. Ouyang W, Ranganath S, Weindel K, Bhattacharya D, Murphy TL, Sha WC and Murphy KM (1998) Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 9: 745–755
30. Bahnson AB, Dunigan J, Baysal B, Mohney T, Atchison RW, Nimgaonkar M, Ball ED and Barranger JA (1995) Centrifugal enhancement of retrovirus-mediated gene transfer. J. Virol. Methods 54: 131–143
31. Grumont RJ, Rourke IJ, O'Reilly LA, Strasser A, Miyake K, Sha W and Gerondakis S (1998) B lymphocytes differentially use the Rel and nuclear factor kappaB1 (NF-κB1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. J. Exp. Med. 187: 663–674
32. Dillon SR, MacKay VL and Fink PJ (1995) A functionally compromised intermediate in extrathymic CD8+ T cell deletion. Immunity 3: 321–333

33. Esslinger CW, Wilson A, Sordat B, Beermann F and Jongeneel CV (1997) Abnormal T lymphocyte development induced by targeted overexpression of IκB alpha. J. Immunol. 158: 5075–5078
34. Attar RM, Macdonald-Bravo H, Raventos-Suarez C, Durham SK and Bravo R (1998) Expression of constitutively active IkappaB beta in T cells of transgenic mice: persistent NF-kappaB activity is required for T cell immune responses. Mol. Cell. Biol. 18: 477–487
35. Senftleben U, Li Z, Baud V and Karin M (2001) IKKbeta Is essential for protecting T cells from TNFα-induced apoptosis. Immunity 14: 217–230
36. Voll RE, Jimi E, Phillips RJ, Barber DF, Rincon M, Hayday AC, Flavell RA and Ghosh S (2000) NF-κB activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. Immunity 13: 677–689
37. Udaka K, Tsomides TJ, Walden P, Fukusen N and Eisen HN (1993) A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8+ T cell clone. Proc. Natl. Acad. Sci. USA 90: 11272–11276
38. Sprent J and Tough DF (1994) Lymphocyte life-span and memory. Science 265: 1 395–1400
39. Tough DF and Sprent J (1994) Turnover of naive- and memory-phenotype T cells. J. Exp. Med. 179: 1127–1135
40. Swain SL, Hu H and Huston G (1999) Class II-independent generation of CD4 memory T cells from effectors. Science 286: 1381–1383
41. Goldrath AW, Bogatzki LY and Bevan MJ (2000) Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. J. Exp. Med. 192: 557–564
42. Cho BK, Rao VP, Ge Q, Eisen HN and Chen J (2000) Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. J. Exp. Med. 192: 549–556
43. Ernst DN, Weigle WO, Noonan DJ, McQuitty DN and Hobbs MV (1993) The age-associated increase in IFN-gamma synthesis by mouse CD8+ T cells correlates with shifts in the frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression. J. Immunol. 151: 575–587
44. Linton PJ, Haynes L, Klinman NR and Swain SL (1996) Antigen-independent changes in naive CD4 T cells with aging. J. Exp. Med. 184: 1891–1900
45. Hurst SD, Sitterding SM, Ji S and Barrett TA (1997) Functional differentiation of T cells in the intestine of T cell receptor transgenic mice. Proc. Natl. Acad. Sci. USA 94: 3920–3925
46. Lee WT, Cole-Calkins J and Street NE (1996) Memory T cell development in the absence of specific antigen priming. J. Immunol. 157: 5300–5307
47. Pihlgren M, Dubois PM, Tomkowiak M, Sjogren T and Marvel J (1996) Resting memory CD8+ T cells are hyperreactive to antigenic challenge *in vitro*. J. Exp. Med. 184: 2141–2151
48. Kashii Y, Giorda R, Herberman R, Whiteside T and Vujanovic N. (1999) Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by NK cells. J. Immunol. 263: 5358–5366
49. Guerder S, Rincon M and Schmitt-Verhulst AM. (2001) Regulation of activator protein-1 and NF-κB in CD8+ T cells exposed to peripheral self-antigens. J. Immunol. 166: 4399–4407
50. Scott DE, Kisch W and Steinberg AD (1993) Studies of T cell deletion and anergy following *in vivo* administration of SEB to normal and lupus-prone mice. J. Immunol. 150: 664–672
51. Senju S, Negishi I, Motoyama N, Wang F, Nakayama K-i, Nakayama K, Lucas PJ, Hatakeyama S, Zhang Q, Yonehara S and Loh DY (1995) Functional significance of the Fas molecule in naive lymphocytes. Intl. Immunol. 8: 423–431
52. Jones RG, Elford A, Parsons M, Wu L, Karwczyk C, Yeh W, Hakem R, Rottapel R, Woodgett JR and Ohashi PS (2002) CD28-dependent activation of protein kinase B/Akt blocks Fas-mediated apoptosis by preventing death-inducing signaling complex assembly. J. Exp. Med. 196: 335–348
53. Garcia S, DiSanto J and Stockinger B (1999) Following the development of a CD4 T cell response *in vivo*: from activation to memory formation. Immunity 11: 163–171
54. Zheng TS, Schlosser SF, Dao T, Hingorani R, Crispe IN, Boyer JL and Flavell RA. (1998) Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis *in vivo*. Proc. Natl. Acad. Sci. USA 95: 13618–13623
55. Mora A, Youn J, Keegan A and Boothby M (2001) NF-kappaB/Rel participation in the lymphokine-dependent proliferation of T lymphoid cells. J. Immunol. 166: 2218–2227
56. Moriggl R, Sexl V, Piekorz R, Topham D and Ihle J (1999) Stat5 activation is uniquely associated with cytokine signaling in peripheral T cells. Immunity 11: 225–230
57. Guttridge DC, Albanese C, Reuther JY, Pestell RG and Baldwin AS (1999) NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell. Biol. 19: 5785–5799
58. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C and Strauss M (1999) NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol. Cell. Biol. 19: 2690–2698
59. Lord JD, McIntosh BC, Greenberg PD and Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and Bcl-x genes through the trans-activation domain of Stat5. J. Immunol. 164: 2533–2541
60. Abbadie C, Kabrun N, Bouali F, Smardova J, Stehelin D, Banderbunder B and Enrietto PJ (1993) High levels of c-Rel expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells *in vitro*. Cell 75: 899–912
61. Connolly JD, Rodgers SE, Clarke P, Ballard DW, Kerr LD, Tyler KL and Dermody TS (2000) Reovirus-induced apoptosis requires activation of transcription factor NF-κB. J. Virol. 74: 2981–2989
62. Beg AA, Sha WC, Bronson RT, Ghosh S and Baltimore D (1995) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 376: 167–170.
63. Beg AA and Baltimore D. (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274: 782–784.
64. Micheau O, Lens S, Gaide O, Alevizopoulos K and Tschopp J (2001) NF-κB signals induce the expression of c-FLIP. Mol. Cell. Biol. 21: 5299–5305
65. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer J, Schroter M, Burns K, Mattmann C, Rimoldi D, French L and Tschopp J (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388: 190–195
66. Van Parijs L, Refaeli Y, Abbas AK and Baltimore D. (1999) Autoimmunity as a consequence of retrovirus-mediated expression of c-FLIP in lymphocytes. Immunity 11: 763–770
67. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli K, Debatin K, Krammer PH and Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 17: 1675–1687
68. Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, Newton K, Tschopp J and Strasser A (1999) Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-xL. Proc. Natl. Acad. Sci USA. 96: 14871–14876
69. De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R and Franzoso G. (2001) Induction of gadd45β by NF-κB downregulates pro-apoptotic JNK signalling. Nature 414: 308–313.
70. Kaltschmidt B, Kaltschmidt C, Hehner SP, Droge W and Schmitz ML (1999) Repression of NF-κB impairs HeLa cell proliferation by functional interference with cell cycle checkpoint regulators. Oncogene 18: 3213–3225
71. Fujii H, Ogasawara K, Otsuka H, Suzuki M, Yamamura K, Yokochi T, Miyazaki T, Suzuki H, Mak TW, Taki S and Taniguchi T (1998) Functional dissection of the cytoplasmic subregions of the IL-2 receptor beta chain in primary lymphocyte populations. EMBO J. 17: 6551–6557
72. Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL and Littman DR (2000) PKC-theta is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. Nature 404: 402–407
73. Coudronniere N, Villalba M, Englund N and Altman A (2000) NF-κB activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta. Proc. Natl. Acad. Sci. USA 97: 3394–3399
74. Seetharaman R, Mora A, Nabozny G, Boothby M and Chen J. (1999) Essential role of T cell NF-κB activation in collagen-induced arthritis. J. Immunol. 163: 1577–1574
75. Aronica M, Mora A, Mitchell D, Johnson J, Finn P, Sheller JR and Boothby M. (1999) Preferential role for NF-κB/Rel signaling in the type 1 but not type 2 T cell-dependent immune response *in vivo*. J. Immunol. 163: 5116–5125