

Perturbation of the T Lymphocyte Lineage In Transgenic Mice Expressing a Constitutive Repressor of Nuclear Factor (NF)- κ B

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Summary

Members of the nuclear factor (NF)- κ B/Rel family transcription factors are induced during thymic selection and in mature T lymphocytes after ligation of the T cell antigen receptor (TCR). Despite these findings, disruption of individual NF- κ B/Rel genes has revealed no intrinsic defect in the development of mature T cells, perhaps reflecting functional redundancy. To circumvent this possibility, the T cell lineage was targeted to express a *trans*-dominant form of I κ B α that constitutively represses the activity of multiple NF- κ B/Rel proteins. Transgenic cells expressing this inhibitor exhibit a significant proliferative defect, which is not reversed by the addition of exogenous interleukin-2. Moreover, mitogenic stimulation of splenocytes leads to increased apoptosis of transgenic T cells as compared with controls. In addition to deregulated T cell growth and survival, transgene expression impairs the development of normal T cell populations as evidenced by diminished numbers of TCR^{hi} CD8 single-positive thymocytes. This defect was significantly amplified in the periphery and was accompanied by a decrease in CD4⁺ T cells. Taken together, these *in vivo* findings indicate that the NF- κ B/Rel signaling pathway contains compensatory components that are essential for the establishment of normal T cell subsets.

Nuclear translocation of members of the nuclear factor (NF)- κ B/Rel transcription factor family is activated in thymocytes and mature T lymphocytes after engagement of the TCR (1–4). The prototypic form of NF- κ B is a heterodimeric complex containing NF- κ B1 (p50) or NF- κ B2 (p52), either of which may combine with a *trans*-activating subunit. The major *trans*-activating subunits of NF- κ B that are induced during T cell activation are c-Rel and RelA (p65) (5). In quiescent T lymphocytes, these various forms of NF- κ B are sequestered in the cytoplasm by virtue of their association with a set of inhibitory proteins that includes I κ B α (6, 7). During normal T cell activation, I κ B α is rapidly degraded via the ubiquitin–proteasome pathway, thus permitting the nuclear import of NF- κ B (6, 7). Binding sites for NF- κ B have been found in many genes involved in T cell effector function, including those that encode regulatory cytokines and receptors (5). These findings raise the possibility that NF- κ B regulates homeostatic mechanisms in the T lineage, such as those involved in cell cycle

control or programmed cell death. Moreover, NF- κ B is induced in developing thymocytes through interactions with stromal cells and during thymic selection (4, 8). Taken together, these *in vitro* studies suggest an important role for NF- κ B/Rel proteins in the development of mature T cells.

Despite these findings, mice deficient for individual NF- κ B/Rel subunits exhibit no intrinsic defect in the establishment of normal populations in the T lineage (9–14). For example, p50-deficient mice express a normal number and distribution of T cells (9). Although mice lacking the RelA subunit of NF- κ B die *in utero*, progenitor cells derived from these animals give rise to a normal T cell repertoire (10, 11). Targeted disruption of the gene encoding RelB, a constitutively expressed member of the NF- κ B/Rel family, leads to a profound reduction in APCs rather than an intrinsic defect in the T lineage (12, 13). In contrast, c-Rel-deficient T cells are impaired in their ability to produce the growth factor IL-2, resulting in decreased proliferation in response to mitogens. Despite this proliferative effect, T cell development in c-Rel^{-/-} mice appears unaffected (14). These unexpected findings have raised unresolved questions concerning whether NF- κ B plays any significant role in the generation of mature T cells (15). However, all of these gene disruption experiments are complicated by the

¹Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; κ B-pd, κ B enhancer sequences based on the IL-2R α promoter; NF- κ B, nuclear factor- κ B; SP, single positive.

potential for functional redundancy. Indeed, prior *in vitro* studies have shown that complexes containing either c-Rel or RelA have the potential to stimulate transcription from the same promoter (16, 17).

To circumvent this critical issue of functional redundancy, we targeted to the T lineage a *trans*-dominant form of I κ B α under tissue-specific control of the proximal I κ C promoter (18, 19). This inhibitor, termed I κ B α (Δ N), lacks sequences required for signal-dependent degradation and functions as a constitutive repressor of multiple NF- κ B/Rel proteins in transfected B and T lymphocytes (6, 7, 18, 20). Consistent with these *in vitro* studies, signal-dependent induction of the c-Rel and RelA *trans*-activating subunits of NF- κ B was profoundly impaired in transgenic thymocytes. This dual block in the NF- κ B/Rel signaling pathway was associated with (a) a proliferative defect that is refractory to IL-2, (b) increased sensitivity of T cells to apoptosis after mitogenic stimulation, and (c) an abnormal distribution of T cell subsets. Taken together, these findings demonstrate that NF- κ B plays a significant role in the development and homeostatic control of mature T cells.

Materials and Methods

Production of Transgenic Mice Expressing I κ B α (Δ N). The cDNA encoding an NH₂-terminally truncated form of human I κ B α (amino acids 37–317) fused to the FLAG epitope (18) was cloned into p1017-I κ C (20). A SpeI fragment encompassing this tissue-specific transcription unit was isolated by agarose gel electrophoresis and micro-injected (Transgenic Core Facility, Vanderbilt Cancer Center, Nashville, TN) into the pronuclei of C57BL/6 \times DBA/2 zygotes together with a BamHI–XbaI fragment containing the locus control region from the human CD2 gene (21). Mice were maintained in accordance with federal and state government regulations after institutional approval. Four independent founders with both fragments integrated in the germline were crossed with C57BL/6 mice for these studies. Northern blot analysis of organ RNAs and immunoblotting with purified B cells confirmed the T lineage specificity of transgene expression (data not shown).

Antibodies and Fluorochrome-conjugated Reagents. Polyclonal antibodies specific for RelA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The c-Rel-specific antiserum was provided by Dr. N. Rice (National Cancer Institute, Bethesda, MD). Rabbit antisera generated against amino acids 289–317 of human I κ B α have been described (18). Fluorochrome-conjugated antibodies against TCR- $\alpha\beta$ (Cy-Chrome), CD3 (r-PE), CD4 (r-PE), CD8 α (FITC or r-PE), CD25 (FITC), CD45R/B220 (FITC), CD69 (FITC), and H-2K^b (FITC) were obtained from PharMingen (San Diego, CA) or GIBCO BRL (Gaithersburg, MD). Avidin and streptavidin-FITC were obtained from PharMingen.

Cell Preparation. Single cell suspensions from thymus, spleen, and lymph node were prepared by crushing the organs in complete media (RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 0.1% penicillin-streptomycin) followed by hypotonic lysis of erythrocytes. PBLs were purified from heparinized blood by density gradient centrifugation on Ficoll-HyPaque (22). Splenic T lymphocytes were depleted of B cells by chromatography through nylon wool columns, followed by panning on immobilized antibodies against mouse IgM (22, 23). In brief, single cell suspensions from pooled spleens were

added to preequilibrated nylon wool columns in RPMI-1640 supplemented with 5% fetal bovine serum at 37°C. After 45 min at 37°C, the nonadherent cells were eluted from the column into plastic Petri dishes (Fisher Scientific) coated with goat anti-mouse IgM (10 μ g/ml) and goat IgG (40 μ g/ml) to remove residual B lymphocytes. The resultant population was <10% B220⁺ and 75–90% T cells as determined by flow cytometry.

Immunoprecipitations and Western Blot Analyses. Cytosolic extracts were prepared from single cell suspensions cultured in the presence or absence of PMA (50 ng/ml) and ionomycin (1 μ M) for 30 min using published methods (18), except that the detergent lysis buffer was supplemented with a mixture of protease inhibitors (24). Where indicated, the translation inhibitor cycloheximide was added at 50 μ g/ml for 30 min before stimulation. Lysates were clarified by centrifugation and equilibrated in ELB buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [Hepes] pH 7.0, 250 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and 0.1% NP40). Immunoprecipitations were performed on cytoplasmic extracts (500 μ g) using 20 μ l of agarose beads coupled to antibodies specific for either RelA, c-Rel, or the FLAG epitope. After three washes with ELB buffer, proteins were either eluted with cognate peptide (RelA, FLAG) or SDS, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Dupont/NEN Life Science, Wilmington, DE). Membranes were blocked (1 h at room temperature) with Tris-buffered saline containing 0.1% Tween-20 and 5% powdered milk (BLOTTO), then incubated with a rabbit antiserum specific for I κ B α . Immunoreactive polypeptides were detected with donkey anti-rabbit IgG conjugated to horseradish peroxidase using enhanced chemiluminescence (Dupont).

Gel Shift Analysis and DNA-Protein Cross-linking. Nuclear fractions were prepared from single cell suspensions by high-salt extraction in the presence of protease inhibitors (18). Gel mobility shift assays were performed by using a ³²P-labeled oligonucleotide duplex derived from κ B enhancer sequences in the IL-2 receptor α promoter (κ B-pd) (5'-CAACGGCAGGGGAATTCCTCTCCTT-3') (24). DNA-binding reaction mixtures (20 μ l) contained 4 μ g of nuclear extract, 2 μ g double-stranded poly (dI-dC), and 10 μ g BSA buffered in 20 mM Hepes (pH 7.9), 5% glycerol, 1 mM EDTA, 1% NP40, and 5 mM dithiothreitol. Resultant nucleoprotein complexes were resolved on a native 5% polyacrylamide gel and visualized by autoradiography (25). For DNA-protein cross-linking analysis, photoreactive derivatives of the radiolabeled κ B probe were synthesized in the presence of 5-bromo-2'-deoxyuridine triphosphate (BrdUTP). DNA binding reaction mixtures were irradiated at 300 nm for 30 min using a Fotodyne UV transilluminator, diluted to 200 μ l with ELB buffer, and immunoprecipitated with the indicated Rel-specific antibodies and protein A-agarose (20 μ l). Immune complexes were washed three times in ELB buffer, heat denatured in SDS sample buffer, resolved by SDS-PAGE, and detected by autoradiography.

Quantitation of IL-2 in Culture Supernatants. Production of IL-2 was measured by ELISA of supernatants from thymocytes and splenocytes after mitogenic stimulation. The ELISAs were performed using plates precoated with anti-mouse IL-2 according to the instructions of the manufacturer (Endogen, Boston, MA). IL-2 levels were quantitated by comparison to standards supplied by the manufacturer.

Proliferation Assays. Thymocyte suspensions were counted and plated (2 \times 10⁵ cells per 100 μ l of media) in microtitre wells pretreated overnight with either PBS or anti-CD3 mAb (10 μ g/ml) (145-2C11, PharMingen; or 29B, GIBCO BRL). Triplicate samples were cultured for 48 h at 37°C in the presence of PMA and

ionomycin or Con A (2.5 $\mu\text{g/ml}$). Tritiated thymidine (1 μCi in 100 μl of media) was added to each well for the final 8 h before determination of radioisotope incorporation into DNA. To quantitate T cell proliferation, pooled spleen and lymph node suspensions were depleted of B cells and macrophages by chromatography through nylon wool columns, then used in proliferation assays, as above, in the presence or absence of an activating hamster mAb against mouse CD28 (10 $\mu\text{g/ml}$; PharMingen, clone 37.51). To distinguish the proliferative capacity of lymphoblasts from small lymphocytes, splenocytes were cultured for 40 h in the absence or presence of Con A (2.5 $\mu\text{g/ml}$) and then treated for 1 h with 5-bromo-2'-deoxyuridine (BrdU; 100 μM). Fractions of BrdU-positive CD4⁺ or CD8⁺ cells were measured as described (26). In brief, after staining with either anti-CD4-rPE or anti-CD8-rPE, cells were permeabilized with 95% EtOH, fixed with paraformaldehyde, and treated with 50 U of DNase I (Boehringer-Mannheim Biochemicals, Indianapolis, IN). After a 10 min incubation at room temperature, cells were stained with a FITC-conjugated mAb against BrdU (Beckton-Dickinson, Mountain View, CA) and analyzed by flow cytometry. Significant BrdU uptake was restricted to cells in the lymphoblast gate as determined by relative light-scattering characteristics (data not shown).

TdT-mediated dUTP-Biotin Nick End Labeling (TUNEL) Assays and Indirect Immunofluorescence. Surface staining of single cell suspensions was performed using optimized concentrations of fluorochrome-conjugated purified mAbs (0.25 μg per 10^6 cells) (27). Samples of $1-2 \times 10^4$ cells were analyzed using a FACScan[®] Plus flow cytometer (Beckton-Dickinson, Mountain View, CA) with uniform gates and quadrants. To generate samples for quantitative TUNEL assays, single cell suspensions were cultured in the presence of either Con A (2.5 $\mu\text{g/ml}$) or immobilized anti-CD3 mAb (10 $\mu\text{g/ml}$). After 40 h of culture, cells were harvested and fractions were stained with PE-conjugated mAb against CD4 or CD8 (PharMingen). To perform TUNEL assays, PE-stained cells were fixed with paraformaldehyde and permeabilized with 70% EtOH at -20°C . After washing in PBS supplemented with 1% BSA (PBS/BSA), cells were resuspended in reaction mixtures containing terminal deoxynucleotidyl transferase (TdT; GIBCO BRL) and biotin-16-dCTP (Boehringer-Mannheim Biochemicals). After incubation at 37°C for 30 min, cells were washed in PBS/BSA, stained with FITC-conjugated avidin, and analyzed by flow cytometry (28). Control reactions lacking TdT were performed to generate a background profile for assessment of nonspecific staining. For restimulation experiments (29), pooled spleen and lymph node cell suspensions were depleted of B cells and macrophages by chromatography through nylon wool columns, cultured for 24 h in the presence of Con A (5 $\mu\text{g/ml}$), washed with methyl- α -D-mannoside (10 mg/ml), then incubated in complete media containing IL-2 (100 $\mu\text{M/ml}$) for an additional 24 h. Viable cells were recovered by fractionation on Ficoll-HyPaque step density gradients and replated (5×10^5 cells/ml) in the presence of immobilized anti-CD3 antibodies (0.005–0.5 $\mu\text{g/ml}$) or mouse TNF- α (1 or 50 ng/ml; R&D Systems, Minneapolis, MN). Cell death was then quantitated using forward light scatter and 7-amino actinomycin D staining characteristics, as described (29).

Results

Inhibition of Nuclear c-Rel and RelA Activity in T Lineage Cells. The induced *trans*-activation of NF- κB -responsive genes in the T lineage is mediated primarily by c-Rel and

RelA (reviewed in 5–7). Prior *in vitro* studies have shown that c-Rel and RelA have the capacity to activate transcription from an overlapping set of promoters (16, 17), thus suggesting compensatory functions. To bypass the potential for functional redundancy, we created transgenic mice expressing a truncated form of I $\kappa\text{B}\alpha$ that is refractory to signal-induced degradation (18). Transgene expression was specifically targeted to the T lineage using the proximal Ick promoter (20), thus facilitating studies to assess the role of the NF- κB /Rel signaling pathway in mouse T lineage cells. Four founder lines expressing this constitutive repressor, termed I $\kappa\text{B}\alpha(\Delta\text{N})$, were classified as Tg^{lo} ($n = 2$) or Tg^{hi} ($n = 2$) based upon the magnitude of transgene expression. The I $\kappa\text{B}\alpha(\Delta\text{N})$ protein was readily detected in both thymus and spleen from transgenic mice, albeit at different apparent steady-state levels in these unfractionated cell populations (Fig. 1 A, lanes 3 and 6). Importantly, this difference was negligible when immunoblotting experiments were performed with comparable numbers of T lineage cells from each organ (data not shown).

To determine whether the I $\kappa\text{B}\alpha(\Delta\text{N})$ protein integrated into the endogenous NF- κB /Rel signaling pathway, co-immunoprecipitation studies were performed with Rel-specific antisera. These experiments revealed the presence of I $\kappa\text{B}\alpha(\Delta\text{N})$ in latent NF- κB /Rel complexes containing the transactivating subunits c-Rel (data not shown) and RelA (Fig. 1 B). Levels of the endogenous form of I $\kappa\text{B}\alpha$ associated with RelA were dramatically decreased; however, prolonged exposure of the immunoblots revealed residual amounts of this inhibitor (Fig. 1 B, lane 3; data not shown). In contrast with wild-type I $\kappa\text{B}\alpha$ (Fig. 1 B, lanes 1 and 2), I $\kappa\text{B}\alpha(\Delta\text{N})$ was resistant to degradation in thymocytes treated with PMA and ionomycin (lanes 3 to 6), a combination that mimics activation through the TCR. To investigate the integrity of the NF- κB /Rel signaling pathway, mobility shift analyses were performed with nuclear extracts from cells treated with PMA and ionomycin. Under these stimulatory conditions, wild-type but not Tg^{hi} thymocytes expressed high levels of nuclear NF- κB /Rel activity (Fig. 1 C, lanes 2 and 6), whereas thymocytes from Tg^{lo} animals expressed intermediate levels of nuclear NF- κB (lane 4). In addition to being dose-dependent, the observed signaling defect was selective, in that induction of AP-1 activity and *c-jun* RNA was unimpaired in transgenic thymocytes (data not shown).

To investigate the subunit composition of the affected complexes, we performed DNA-protein cross-linking experiments. These studies revealed a striking decrease in nuclear RelA and c-Rel in extracts from Tg^{hi} mice as compared with nontransgenic (NTg) controls (Fig. 1 D, lanes 2 and 6). Similar results were obtained using antibodies specific for c-Rel and RelA in supershift analyses (data not shown). In keeping with these results using Tg^{hi} thymocytes, nuclear expression of inducible NF- κB complexes was also decreased in splenic T cells from Tg^{hi} mice when compared with NTg controls (Fig. 1 E, lanes 6 and 8). Taken together, these findings indicate that I $\kappa\text{B}\alpha(\Delta\text{N})$ spe-

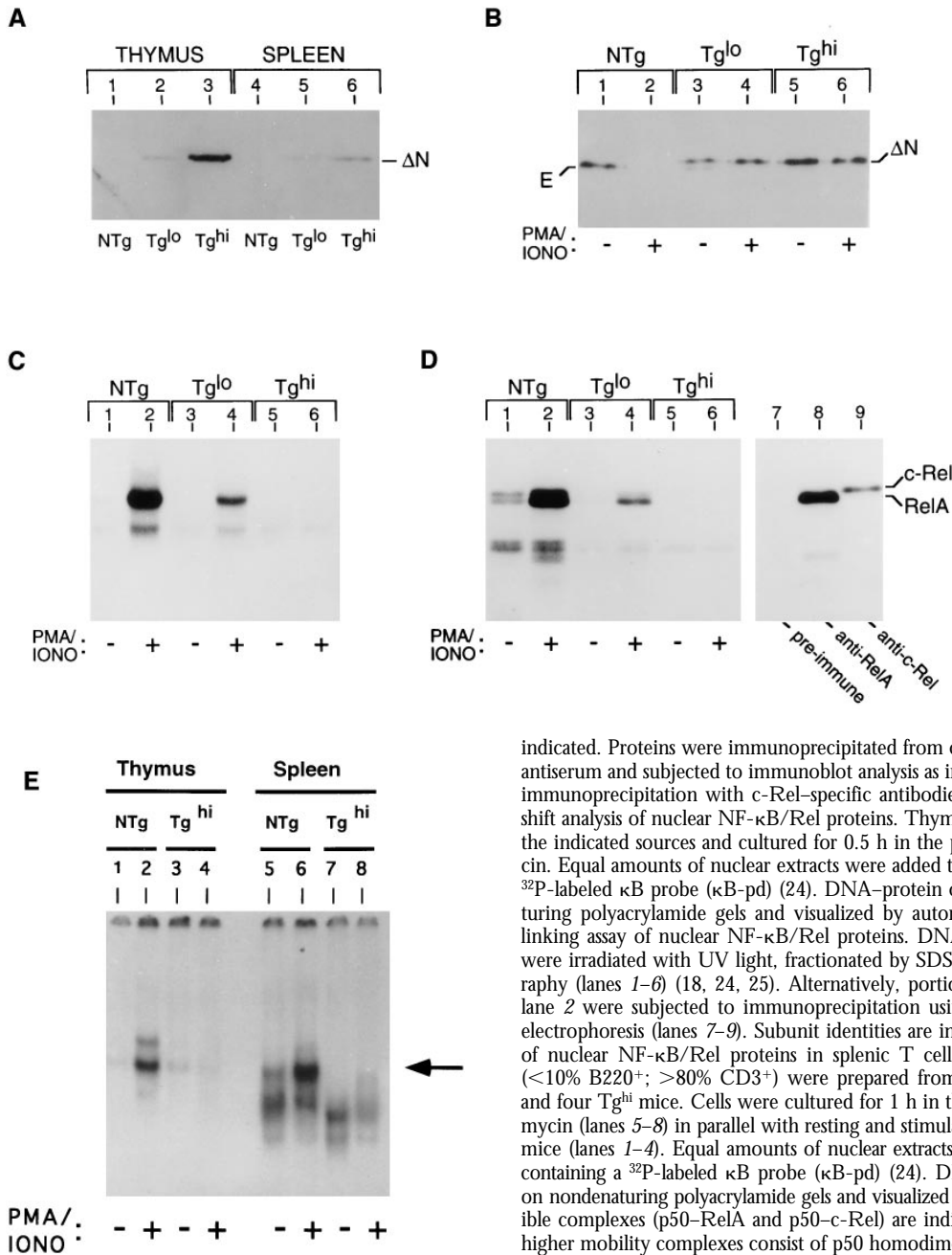


Figure 1. Abrogated expression of NF- κ B/Rel in I κ B α (Δ N) transgenic mice. (A) Transgene-encoded I κ B α expressed in thymocytes and splenocytes. Cytoplasmic extracts from unfractionated cell suspensions were subjected to immunoprecipitation using immobilized anti-FLAG M2 antibodies and resolved by SDS-PAGE. Resolved proteins were transferred to polyvinylidene difluoride membranes and probed with an antiserum directed against human I κ B α (amino acids 289–317) in conjunction with an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) (18). NTg, nontransgenic; Tg^{lo}, low expressor; Tg^{hi}, high expressor. (B) Association of endogenous I κ B α (E) and transgene-encoded I κ B α (Δ N) with the RelA subunit of NF- κ B. Isolated thymocytes from the indicated sources were cultured for 0.5 h in the presence of cycloheximide (50 μ g/ml) to arrest translation and then treated with PMA and ionomycin for 0.5 h as

indicated. Proteins were immunoprecipitated from cytosolic extracts using a RelA-specific antiserum and subjected to immunoblot analysis as in A. Similar results were obtained after immunoprecipitation with c-Rel-specific antibodies (data not shown). (C) Gel mobility shift analysis of nuclear NF- κ B/Rel proteins. Thymocyte suspensions were prepared from the indicated sources and cultured for 0.5 h in the presence or absence of PMA/ionomycin. Equal amounts of nuclear extracts were added to DNA binding mixtures containing a ³²P-labeled κ B probe (κ B-pd) (24). DNA-protein complexes were resolved on nondenaturing polyacrylamide gels and visualized by autoradiography. (D) DNA-protein cross-linking assay of nuclear NF- κ B/Rel proteins. DNA binding reaction mixtures (as in C) were irradiated with UV light, fractionated by SDS-PAGE, and visualized by autoradiography (lanes 1–6) (18, 24, 25). Alternatively, portions of reaction mixtures generated for lane 2 were subjected to immunoprecipitation using the indicated antiserum before gel electrophoresis (lanes 7–9). Subunit identities are indicated. (E) Gel mobility shift analysis of nuclear NF- κ B/Rel proteins in splenic T cells. B cell-depleted T cell populations (<10% B220⁺; >80% CD3⁺) were prepared from splenocytes pooled from three NTg and four Tg^{hi} mice. Cells were cultured for 1 h in the presence or absence of PMA/ionomycin (lanes 5–8) in parallel with resting and stimulated thymocytes pooled from the same mice (lanes 1–4). Equal amounts of nuclear extracts were added to DNA binding mixtures containing a ³²P-labeled κ B probe (κ B-pd) (24). DNA-protein complexes were resolved on nondenaturing polyacrylamide gels and visualized by autoradiography. The major inducible complexes (p50-RelA and p50-c-Rel) are indicated with an arrow; the constitutive, higher mobility complexes consist of p50 homodimers (30).

cifically represses signal-dependent activation of nuclear RelA and c-Rel, which are normally induced during thymic selection and T cell activation (4, 8, 30).

Decreased IL-2 Production by Transgenic Thymocytes and T Cells. Prior *in vitro* studies have suggested a central role for NF- κ B/Rel proteins in transcriptional regulation of the IL-2 gene (31, 32). Consistent with these findings, IL-2 production is impaired in T cells derived from c-Rel-deficient mice (14). Considering the inhibitory effects of I κ B α (Δ N) on c-Rel DNA binding activity, we next investigated whether IL-2 gene expression was compromised in transgenic thymocytes. For these studies, thymocytes from Tg^{hi} mice and their NTg littermates were cultured in the

absence or presence of PMA and ionomycin, followed by ELISA to quantitate the concentration of IL-2 secreted into culture supernatants. As shown in Fig. 2, wild-type thymocytes produced 14,100 pg/ml of IL-2, reflecting at least a 3,000-fold increase in cytokine expression relative to the basal level. In contrast, IL-2 production by comparable numbers of activated Tg^{hi} thymocytes was drastically reduced to 645 pg/ml. In companion studies, we found that IL-2 production by mitogen-stimulated Tg^{hi} splenocytes was also attenuated relative to controls (8% of control; data not shown). These findings demonstrate that the block imposed on the DNA binding activities of c-Rel and RelA (see Fig. 1) is associated with a profound deficit in IL-2

production, thus establishing the presence of a significant functional defect in the NF- κ B/Rel signaling pathway that affects the expression of downstream genes.

Inhibition of Growth Signal Transduction by $I\kappa B\alpha(\Delta N)$. Prior studies have shown that c-Rel-deficient T cells develop normally but exhibit impaired mitogenic responses (14). This proliferative defect was fully reversed by exogenous IL-2 (14). Evidence of a transgene-dependent block in IL-2 production by Tg^{hi} thymocytes led us to investigate their proliferative response to the T cell mitogens Con A, combinations of PMA and ionomycin, or immobilized antibody to the TCR (anti-CD3). As shown in Fig. 3, Tg^{hi} thymocytes manifested a dramatic decrease in the proliferation induced by each of these mitogens (A–C; control). We reasoned that this growth defect might be attributed to the 20-fold difference in IL-2 concentrations in these cultures (see Fig. 2). However, as shown in Fig. 3 A addition of exogenous IL-2 to Tg^{hi} thymocytes failed to restore proliferation to normal levels. Similar results were obtained with unfractionated (data not shown) or B cell-depleted splenocytes (Fig. 3 D). These results are in sharp contrast with those obtained using c-Rel-deficient mice (14), presumably reflecting an additional contribution by RelA (Fig. 1 D). In this regard, there was a 50% decrease in the frequency of T cells positive for IL-2R α chain (CD25), which has in its 5'-flanking DNA a site for RelA/p50 binding (5, 33). However, Tg^{hi}-derived cells that were able to undergo blast transformation and enter S phase expressed normal levels of CD25 (data not shown). We conclude that the failure of exogenous IL-2 to restore normal proliferation of Tg^{hi} T cells is likely due to the reduced expression of a competence factor other than the IL-2R α chain.

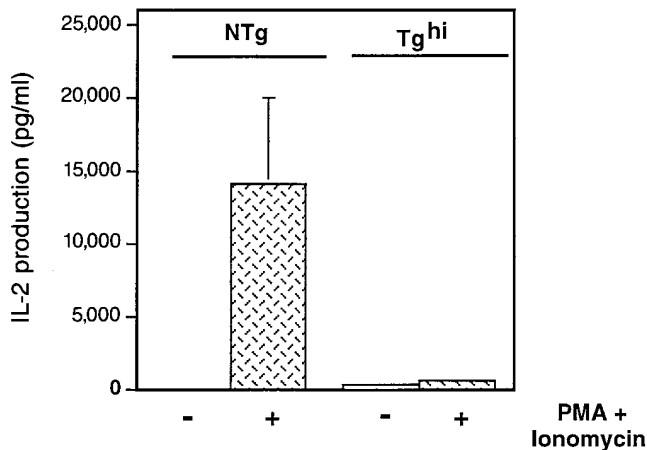


Figure 2. IL-2 production by thymocytes from $I\kappa B\alpha(\Delta N)$ transgenic mice. Thymocytes from either NTg or Tg^{hi} mice were cultured in triplicate for 48 h in media alone (open bars) or treated with combinations of PMA (50 ng/ml) and ionomycin (1 μ g/ml) (stippled bars). Supernatants from these cultures and IL-2 standards were assayed by ELISA. IL-2 production by unstimulated NTg thymocytes was below the detection limit of the assay (3–5 pg IL-2/ml). Results represent the mean values (\pm SEM) from five separate Tg^{hi} mice and five NTg littermates, as analyzed in three separate experiments.

Enhanced Apoptosis of Rel-arrested T Cells After Activation. It is now well recognized that programmed cell death can occur in response to primary stimulation through the TCR (34–36) or after activated T cells are exposed to certain proapoptotic agents (37). Moreover, evidence from studies with established cell lines suggests that NF- κ B may either inhibit (38, 39) or enhance their susceptibility to apoptosis (40–42). Accordingly, we next investigated the influence of $I\kappa B\alpha(\Delta N)$ transgene expression on entry into the apoptotic pathway after primary activation of resting T cells with mitogens. When the prevalence of apoptotic T cells was determined by TUNEL assays after mitogenic stimulation with Con A, we observed that expression of the $I\kappa B\alpha(\Delta N)$ transgene led to a two-fold increase in the frequency of T cell apoptosis (Fig. 4 A). This enhancement in programmed cell death applied to both CD4⁺ and CD8⁺ T cells. Similar results were obtained using immobilized anti-TCR antibodies as the stimulus, albeit with a more pronounced effect on the CD8⁺ subset (Fig. 4 B). Selective gating on lymphoblasts revealed a doubling in the frequency of apoptotic CD8⁺ T cells derived from the transgenic mice as compared with controls. Thus, the increased sensitivity to apoptosis observed under these experimental conditions cannot be attributed solely to the failure of these cells to undergo blast transformation. We conclude that the expression of $I\kappa B\alpha(\Delta N)$ in transgenic T cells is associated with enhanced apoptosis of T cells in response to primary TCR stimulation (35, 36).

$I\kappa B\alpha(\Delta N)$ Perturbs Development of Mature T Cell Lineages. In contrast with c-Rel-deficient mice, our results with $I\kappa B\alpha(\Delta N)$ transgenic animals demonstrate a proliferative defect that is refractory to IL-2, as well as enhanced T cell apoptosis. These novel defects raised the possibility that establishment of T cell populations might be abnormal in $I\kappa B\alpha(\Delta N)$ transgenic mice. To investigate this possibility, we first measured the prevalence of CD4 and CD8 single-positive (SP) cells among thymocytes. Thymic cellularity was unaffected by the transgene and FACS[®] profiles indicated normal frequencies of CD4⁻CD8⁻ and CD4⁺CD8⁺ thymocytes (Fig. 5 A). Thus, despite significant inhibition of NF- κ B/Rel activity (see Fig. 1 C), TCR α and β chain gene rearrangement proceed normally, as does the expansion of immature thymocytes to the double-positive stage (43). In contrast, Tg^{hi} CD8 SP thymocytes bearing high levels of TCR- $\alpha\beta$ were decreased 40% relative to NTg controls ($P < 0.0001$), whereas TCR^{hi} CD4 SP cells were unaffected (Fig. 5 B). There was no difference in the staining intensity for CD8 present on any thymocyte subset, which excludes the possibility that $I\kappa B\alpha(\Delta N)$ downregulated the CD8 gene. Furthermore, expression of the class I MHC antigen K^b on Tg^{hi} thymocytes and splenocytes was normal, as were mRNA levels for the H-2K antigen. As such, the observed decrease in CD8 SP cells does not appear to be due to inadequate class I MHC expression (44, 45). Given that the TCR^{hi} subset of thymocytes is associated with repertoire selection (46), we conclude that T cell development in the thymus of Tg^{hi} mice is partially impaired.

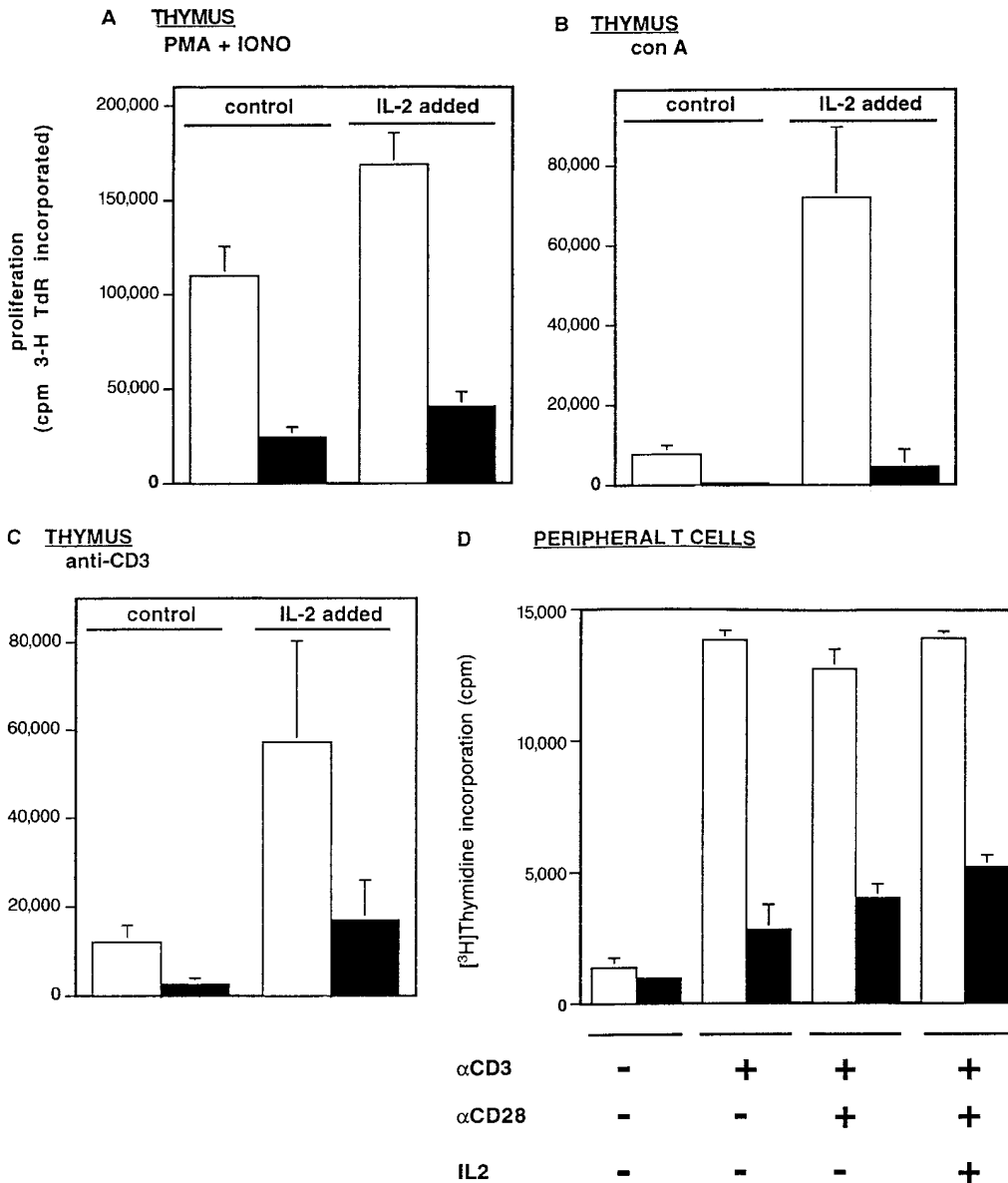


Figure 3. Failure of IL-2 to rescue the proliferative defect in $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice. Thymocytes from either NTg (open bars) or Tg^{hi} (closed bars) mice were treated for 40 h with (A) combinations of PMA (50 ng/ml) and ionomycin (1 $\mu\text{g}/\text{ml}$). (B) Con A (2.5 $\mu\text{g}/\text{ml}$), or (C) plate-bound anti-CD3 (10 $\mu\text{g}/\text{ml}$), in the presence or absence of IL-2 (100 U/ml) as indicated. Cells were pulsed for an additional 8 h with tritiated thymidine and harvested for scintillation counting. The results are shown as the mean of tritiated thymidine incorporation (\pm SEM) for eight Tg^{hi} mice and eight NTg littermates (four independent experiments). (D) Splenocytes depleted of B cells and macrophages were analyzed under similar conditions using plate-bound anti-CD3 (10 $\mu\text{g}/\text{ml}$), agonistic antibodies against CD28 (10 $\mu\text{g}/\text{ml}$), and IL-2 (100 U/ml) as indicated.

While T cell subsets are established during the differentiation of immature precursors in the thymus, the ultimate outcome of T cell development is the establishment of normal populations in the periphery (47). To investigate further the significance of NF- $\kappa\text{B}/\text{Rel}$ proteins in this process, we measured the prevalence of T cells in spleen, lymph node, and blood. As shown in Fig. 6 A, T cell numbers were significantly reduced at each of these peripheral sites in Tg^{hi} animals as compared with NTg littermates. With respect to mature T cell subsets, CD8^+ T cells were substantially decreased in the periphery of Tg^{hi} animals relative to controls (Fig. 6 B). A significant loss of CD4^+ cells was also

observed in spleen and blood, although this defect was consistently less severe (Fig. 6 C). Similar evidence for skewing of $\text{CD4}/\text{CD8}$ ratios was obtained by gating specifically on α/β -TCR-bearing cells in the spleen, lymph nodes, and blood (data not shown). Thus, the asymmetric effect of $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ on the populations of positively selected SP cells in the thymus is amplified in the periphery. An altered ratio of $\text{CD4}/\text{CD8}$ subsets was also observed in the Tg^{lo} lines of mice. However, consistent with the biochemical data (see Fig. 1 C), these CD8^+ T cells were less profoundly affected (50% of normal numbers) relative to Tg^{hi} mice (Fig. 6, legend), demonstrating a dose-dependent effect of the trans-

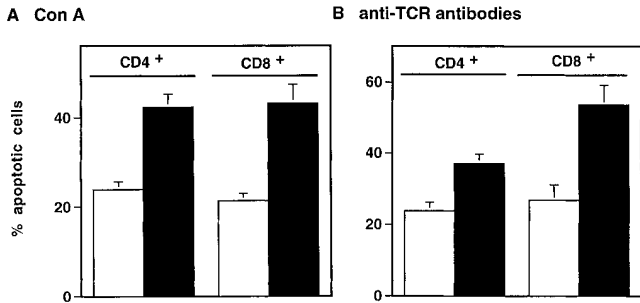


Figure 4. Increased apoptosis among activated T cells from $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice. (A) Splenocytes from either NTg (open bars) or Tg^{hi} (closed bars) mice were cultured in the presence of Con A (2.5 $\mu\text{g}/\text{ml}$). After 40 h in culture, cells were divided equally, stained with PE-labeled antibodies against CD4 or CD8, and subjected to TUNEL analysis. The data represent the mean percentage (\pm SEM) of TUNEL-positive cells in each T cell subset. The mean was calculated using data from 19 individual Tg^{hi} mice or an equal number of NTg controls (10 independent experiments with 6–8-wk-old mice). The increased frequencies of apoptotic cells among transgenic CD4^+ and CD8^+ cells were statistically significant when compared with nontransgenic CD4^+ or CD8^+ cells ($P < 0.001$). (B) Splenocytes were activated using plate-bound anti-CD3, then processed for TUNEL assays as in A. Results represent cumulative data from 12 individual Tg^{hi} mice or an equal number of NTg controls (six independent experiments with 6–8-wk-old mice). The increased frequencies of apoptotic cells among transgenic CD4^+ and CD8^+ cells were statistically significant when compared with nontransgenic CD4^+ or CD8^+ cells ($P \leq 0.001$).

gene. Because endogenous $\text{I}\kappa\text{B}\alpha$ is still present in this transgenic system (see Fig. 1 B), our results may underestimate the quantitative significance of NF- κB /Rel proteins in determining T cell levels. Notwithstanding this uncer-

tainty, these findings provide strong in vivo evidence that an intact NF- κB /Rel signaling pathway is essential for acquisition of a normal proportion of CD8^+ T cells.

Discussion

Since its original discovery in B cells, NF- κB has been widely implicated in the transcriptional control of genes involved in T cell activation and growth (5). This signal transduction pathway is tightly coupled to the TCR and is induced during selection of double-positive thymocytes (1–4, 8). Despite these findings, mice deficient for p50, c-Rel, or RelB have a normal number and distribution of T cell subsets (9–14), thus raising questions concerning the significance of NF- κB /Rel proteins in the establishment and maintenance of a normal T cell compartment (15). However, these gene disruption experiments are complicated by the potential for functional redundancy and, as shown for RelA, can lead to early embryonic lethality (10). To circumvent these technical problems, we targeted to the T lineage a truncated form of $\text{I}\kappa\text{B}\alpha$ that constitutively represses the nuclear expression of multiple NF- κB dimers. When expressed in transgenic thymocytes, this modified inhibitor dramatically attenuated nuclear import of RelA and c-Rel (Fig. 1, C and D). These two Rel-related polypeptides represent the principal *trans*-activating subunits of NF- κB that are induced in response to TCR ligation (1, 5, 8, 30). Based on these biochemical findings, we investigated whether simultaneous arrest of RelA and c-Rel in the T cell compartment is associated with novel phenotypic alterations relative to those reported in prior gene targeting studies.

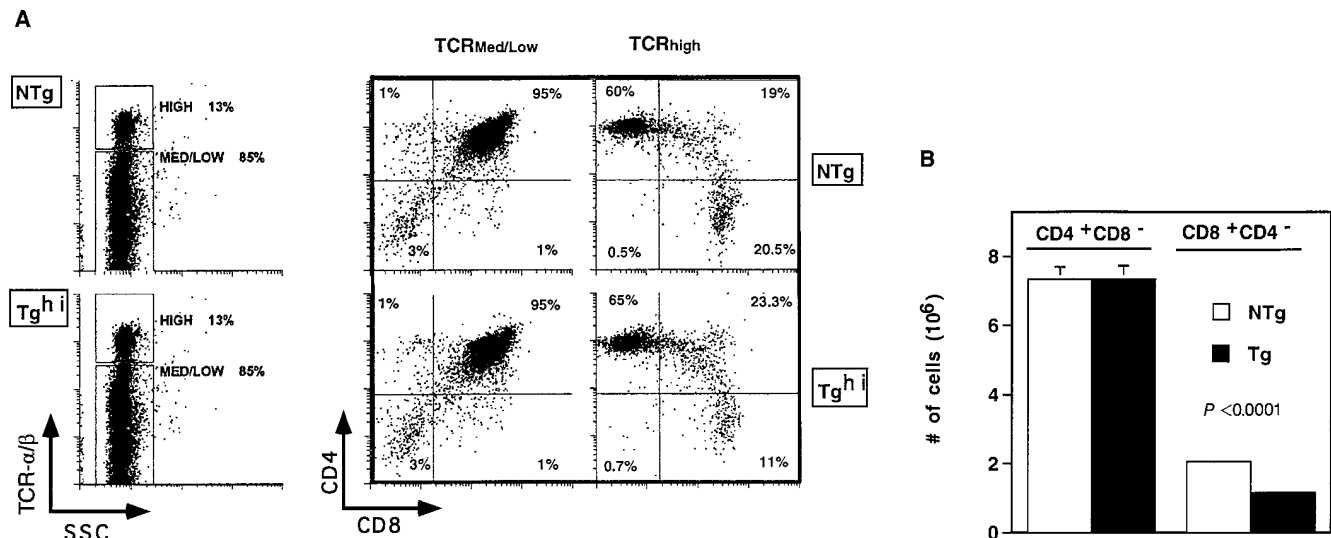
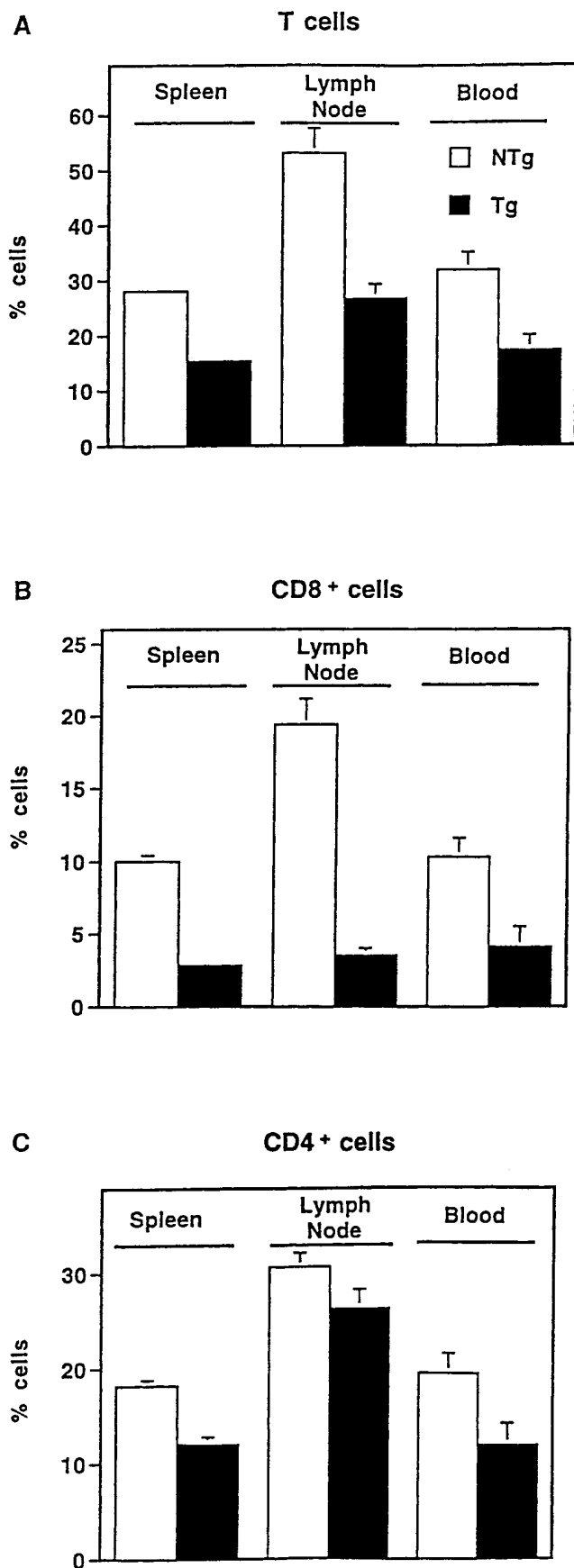


Figure 5. Decreased thymic CD8^+ cells in $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice. (A) A representative FACS[®] profile from three-color indirect immunofluorescence experiments. Thymocytes from NTg and Tg^{hi} mice were analyzed by flow cytometry for surface expression of CD4 and CD8 on cells bearing high density TCR- α/β (TCR^{high}) or medium to low density receptors ($\text{TCR}^{\text{med/low}}$). Gating of cells based on TCR- α/β expression is shown to the left, together with the percentage of total cells in each gate. Dual parameter fluorescence histograms (CD4 versus CD8) derived from the gated subsets ($\text{TCR}^{\text{med/low}}$ and TCR^{high} , respectively) are shown on the right. Numbers represent the percentage of gated thymocytes in each quadrant. Mean cell counts were equivalent in NTg and Tg^{hi} thymuses ($99 [\pm 9.1] \times 10^6$ versus $97.7 [\pm 10.1] \times 10^6$ respectively). (B) Subset distribution of the TCR^{hi} thymocyte populations in NTg (open bars) and Tg^{hi} (closed bars) mice. Mean cell populations (\pm SEM) of TCR^{hi} cells in the CD4^+ CD8^- and CD4^- CD8^+ quadrants are shown ($n = 10$). There was no significant difference in mean numbers of CD4^- CD8^- or CD4^+ CD8^+ cells.



Requirement for RelA and c-Rel in IL-2-responsive Proliferation. During the process of T cell activation, nuclear translocation of NF- κ B is associated with transcriptional activation of the genes encoding IL-2 and IL-2R α (CD25), which together are required for normal growth signal transduction (48–50). Recent gene disruption experiments have demonstrated that c-Rel-deficient T cells exhibit a defect in IL-2 production, which leads to a diminished proliferative response (14). In these prior studies, exogenous IL-2 restored T cell proliferation, indicating that the IL-2 signaling pathway is intact in c-Rel-deficient lymphocytes (14). Although the mitogenic response of T lineage cells expressing I κ B α (Δ N) is also impaired, we have found that this response cannot be fully restored by exogenous IL-2. Given the biochemical differences between the two in vivo systems, these data suggest that RelA contributes a unique function(s) in the T cell activation program that is not compensated for by c-Rel. One potential function involves the control of IL-2R α gene expression, which is normal in c-Rel^{-/-} mice (14). Indeed, prior in vitro studies have suggested that the activity of the IL-2R α promoter is under RelA control (51). However, we have found that T lymphoblasts derived from Tg^{hi} mice express normal levels of surface IL-2R α and that thymocytes from these animals respond to IL-2-mediated growth signals, albeit at lower levels. Taken together, these data suggest that T cells in our experimental system acquire functional IL-2 receptors, thus raising the possibility that NF- κ B regulates genes important for growth signal transduction in T cells following engagement of the IL-2R (52, 53).

Enhanced Apoptosis of Activated T Cells. In addition to growth signal transduction, prior studies have suggested that members of the NF- κ B/Rel family of proteins contribute to the regulation of programmed cell death. For example, high levels of c-Rel expression are associated with apoptosis in the developing avian embryo and bone marrow cells (40). In contrast, more recent studies indicate that NF- κ B protects embryonic fibroblasts and transformed T cells from TNF- α -induced apoptosis (38, 39). The data pre-

Figure 6. Altered T cell subsets in I κ B α (Δ N) transgenic mice. Cells from the indicated peripheral sites were stained with fluorochrome-conjugated mAbs against CD3 (A), CD8 (B), or CD4 (C) and analyzed by flow cytometry. Mean cell counts were equivalent in NTg and Tg^{hi} spleens (77.9 ± 7.2 versus $72.7 \pm 5.0 \times 10^6$ cells, respectively; $P > 0.5$), and reduced in Tg^{hi} lymph nodes ($9.25 \pm 1.6 \times 10^6$ cells) relative to NTg samples ($15.0 \pm 2.6 \times 10^6$ cells; $P = 0.09$). Therefore, the data are presented as the percentage of cells (\pm SEM) expressing these surface markers in spleen ($n = 22$), lymph node ($n = 11$), or blood ($n = 9$). Differences between NTg (open bars) and Tg^{hi} (closed bars) samples were significant at $P < 0.0001$ (spleen) and $P < 0.001$ (blood, lymph node) with the exception of CD4⁺ lymph node cells. Analysis of T cells at each of these sites failed to reveal the CD8^{lo} population previously linked to superantigen-induced apoptosis and CD8⁺ T cell loss (26). Evidence of T cell hyperactivation (50) was absent. Splenic cellularity was normal in two independent Tg^{lo} lines ($n = 9$). CD4⁺ and CD8⁺ cells represented $17.5 \pm 1.6\%$ and $5.0 \pm 0.8\%$ of Tg^{lo} splenocytes (CD4/CD8 ratio = 4.6 ± 0.9). In contrast, CD4⁺ and CD8⁺ cells represented $20.1 \pm 1.0\%$ and $10.0 \pm 0.55\%$ of splenocytes from littermate controls. The observed differences in CD8⁺ cell numbers and CD4/CD8 ratios were significant at $P < 0.001$ and $P < 0.05$, respectively.

sented here indicate that NF- κ B can also protect primary T lymphocytes from death after stimulation through the TCR. These findings may be related to recent studies demonstrating that CD3 stimulation leads to rapid T cell apoptosis in vivo (35, 36). Of note, apoptosis can also be induced by secondary stimulation of T cells activated by treatment with a mitogen and IL-2 (29, 37, 54). Consistent with our primary stimulation data (Fig. 4), we have found that nylon wool-purified T cells from transgenic spleens exhibit enhanced apoptosis using this secondary stimulation model (data not shown). However, Tg^{hi} T cells also exhibit a proliferative defect that cannot be rescued by IL-2 (Fig. 3), a finding that complicates the interpretation of any results obtained using these latter experimental conditions.

In light of the observed steady-state decrease in Tg^{hi} T cell populations (Fig. 6), the NF- κ B-dependent mechanism of protection described here may exert a significant influence on lymphocyte homeostasis by inducing the expression of one or more downstream genes that affect T cell survival. In this regard, the expression of FasL and anti-apoptotic gene products such as Bcl-2 and Bcl-x_L has been linked to the regulation of T cell population size (29, 35, 36, 54–58). However, levels of Bcl-2, Bcl-x, and FasL RNA in resting and stimulated cells from Tg^{hi} and NTg animals were comparable (data not shown). Prior in vitro studies have also shown that the death of activated T cells may result from IL-2 withdrawal or inadequate costimulation through cell surface CD28 (54, 58, 59). However, our preliminary studies indicate that TCR-mediated apoptosis of transgenic T cells cannot be reversed by either exogenous IL-2 or agonistic antibodies directed against CD28 (data not shown). As such, the mechanism by which NF- κ B protects normal T cells from TCR-induced apoptosis may involve a distinct pathway.

Role of NF- κ B in T Cell Development. The thymic phase of T cell development involves an orderly progression from double-negative to double-positive thymocytes, which then undergo selection and emigration. In this biologic context, our data provide three new lines of evidence for the involvement of NF- κ B in T cell development. First, the number of TCR^{hi} CD4⁻CD8⁺ cells in the thymus was decreased, despite the unabated accumulation of immature thymocytes. Second, mature T cell subsets were further compromised in the periphery. Finally, an unexpected but striking finding to emerge from these studies was an asymmetric effect of the transgene on the deployment of CD4⁺ and CD8⁺ cells.

There are very few precedents for a signal transduction pathway which preferentially potentiates development of mature CD8⁺ T cells (60–63). We consider it unlikely that the observed asymmetry is due to subset-specific differences in transgene expression, because RT-PCR and immunoblotting experiments revealed comparable steady-state expression of I κ B α (Δ N) in the CD4⁺ and CD8⁺ lineages (data not shown). Moreover, transgenic thymocytes exhibited a dramatic reduction (>95%) in the synthesis of IL-2 (Fig. 2), which is produced almost exclusively by the CD4⁺ CD8⁻ subset (3, 64, 65). Consistent with this finding, we have also found that transgenic splenocytes produce reduced levels of IL-2 relative to controls (8% of control; data not shown). Finally, NF- κ B induction was arrested in a predominantly CD4⁺ population of Tg^{hi} T cells (Fig. 1 E). These data strongly suggest that the I κ B α (Δ N) transgene blocks NF- κ B signaling in the CD4⁺ T cell compartment. Despite these findings with CD4⁺ cells, transgene expression led to a preferential reduction in CD8⁺ T cell numbers in the thymus and periphery (Figs. 5 and 6). We conclude that the function of the NF- κ B/Rel signaling pathway in lymphocyte homeostasis may be polarized with respect to the deployment of specific T cell subsets. Although the mechanism involved remains unclear, one possibility is that CD8⁺ cells in the intact animal are more dependent on NF- κ B for protection against apoptosis (Fig. 4 B).

In summary, we have found that corepression of RelA and c-Rel in transgenic mice leads to multiple defects in the T lineage. Specifically, transgenic T cells expressing I κ B α (Δ N) exhibited decreased IL-2 production, a proliferative defect that is refractory to exogenous IL-2, and enhanced apoptosis following mitogenic stimulation. Whereas the double-negative and double-positive thymocyte subsets in these mice appeared normal, numbers of TCR^{hi} CD8 SP thymocytes were significantly decreased. This deficit in CD8⁺ T cells was exacerbated in the periphery and accompanied by a decrease in CD4⁺ T cells and skewed CD4/CD8 ratio. These findings were not apparent in prior gene targeting studies (9–14), thus suggesting that RelA and c-Rel serve compensatory functions in homeostasis. Taken together, the data provide clear evidence for the involvement of NF- κ B/Rel proteins in T lineage development. Furthermore, the observed asymmetric population dynamics suggest that the mechanisms controlling CD4⁺ and CD8⁺ T cell numbers may involve distinct NF- κ B signaling requirements.

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