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c-Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function

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Abstract

The TCR signals are essential for T cell activation and proliferation, primarily through the induction of cytokine and cytokine receptors. Several transcription factor families, including NF-κB/Rel, have been implicated in the regulation of cytokine gene expression in T cells in response to antigen, cytokine and mitogenic stimulation. In this study, we show that the mice with a null mutation in the lymphoid-specific c-Rel gene have normal development of lymphoid tissues and T cell compartment. However, T cells derived from the c-Rel knockout mice have several functional abnormalities. The c-Rel-deficient T lymphocytes fail to respond to activation and proliferation signals mediated by the TCR and mitogens *in vitro*. This is attributed to an impaired production of cytokines IL-2, IL-3 and granulocyte macrophage colony stimulating factor. In addition, the induction of IL-2Rα chain is impaired in the c-Rel$^{-/-}$ T cells. The poor expression of cytokines and IL-2Rα chain correlates with a reduced nuclear translocation of NF-κB components in c-Rel$^{-/-}$ T cells. Since activation is prerequisite for differentiation into effector cells, c-Rel$^{-/-}$ T cells failed to differentiate into cytotoxic T cells or T_h cells without rescuing cytokines. However, upon supplement with exogenous IL-2, the c-Rel$^{-/-}$ cytotoxic T lymphocytes are able to execute cytotoxicity and the c-Rel$^{-/-}$ T_h cells are capable of providing help to normal B cells. These data suggest that c-Rel is important for inducible cytokine and cytokine receptor expression, and a key regulator of early activation and proliferation in T cells.

Introduction

The NF-κB/Rel transcription factor family is composed of five members of interacting proteins, c-Rel, p50, p65, p52 and RelB. They share a highly conserved Rel-homology domain which confers the ability to bind DNA, dimerize and interact with the specific cytoplasmic inhibitors, the IκBs (1–8). Early *in vitro* studies in various cell types demonstrated that NF-κB/Rel transcription factors respond to a variety of stimuli. Those relevant to the immune system include antigen receptors on B and T cells, CD40, CD28, IL-1, tumor necrosis factor (TNF)-α, phytohemagglutinin, and lipopolysaccharide as well as other mitogens. Studies on NF-κB/Rel knockout mice confirmed general features of impaired immunity with distinct phenotypes (9–15) and were consistent with the observations that each member has distinct tissue distribution as well as target gene specificities (16).

The activation of T cells is a complex process that has been ascribed to three distinct steps. The first step involves signals transduced by the TCR, while the second step is supplied by accessory, co-stimulatory molecules, principally CD28. Together, signals emanating from these receptors synergize to activate the cell to move from a quiescent, resting G_0 phase into the early, G_1 phase of the cell cycle. The third step is triggered by IL-2, which promotes progression through G_1 into the S phase (17,18).

In the dissection of the molecular events responsible for these steps, the regulation of the genes expressed as a consequence of the first two steps has been studied extensively and, thus far, perhaps the gene studied most thoroughly is the IL-2 gene. Exhaustive analysis of the enhancer region 5′ upstream of the transcriptional start site has identified response elements for several well-characterized transcription factors. These include elements capable of binding AP-1 complexes, NF-AT, NF-κB/Rel and Oct-1 (19–24). Of these various response elements and transcription factors, NF-AT has received the most attention as a key regulator of TCR-dependent activation of IL-2 gene expression, primarily

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because the immunosuppressive drug cyclosporin A has been shown to suppress TCR-promoted T cell proliferation by preventing NF-AT activation of IL-2 gene expression. By comparison with NF-AT, the importance of the NF-κB/Rel family of transcription factors in the regulation of IL-2 gene expression has remained obscure. However, the suppressive effect of glucocorticoids on T cell proliferation has recently been attributed to the induction of IκB expression (25,26). The increase in IκB results in the prevention of NF-κB/Rel activation and a marked inhibition of IL-2 gene expression upon TCR triggering. Supporting the role of NF-κB/Rel in cytokine gene expression, targeted deletion of the c-Rel gene results in a profound deficiency in TCR-triggered IL-2, IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) cytokine production (13,27).

To investigate further the role of c-Rel in the regulation of T cell activation and effector function, we utilized gene targeting to disrupt the c-Rel gene. The absence of c-Rel expression results in a marked deficiency in the capacity of T cells to proliferate in response to T cell mitogens, thus confirming the results of Kontgen *et al*. (13,27). Although this deficiency is primarily attributable to the lack of IL-2 production, the expression of the IL-2Rα chain gene was also found to be depressed compared to wild-type controls. As cytokines and receptors are key mediators of cellular and humoral immune responses, we also tested the effector cell function in c-Rel-deficient T lymphocytes.

Methods

Gene targeting deletion of the c-Rel locus

Several overlapping phage clones which cover 20 kb of the c-Rel genomic locus were isolated from a 129/sv genomic library and were provided by Dr William Sha. The 3.0 kb *Bsp*HI restriction fragment (B3), containing exon 4, was subcloned into the blunted *Eco*RI site of the pNT-neo vector (28) to generate the pNT–B3 construct. The *Eco*RV 7.6 kb fragment, containing exons 6 and 7, was then subcloned into the blunted *Xho*I site in the pNT-B3 to generate the final c-Rel targeting construct, pNT–B3-RV7.6. This construct was transfected into the embryonic stem cell line, J1, kindly provided by Dr En Li (29), and selected with 180 μg/ml G418 and 2 μM ganciclovir. To screen for homologous recombination, genomic DNA from several hundreds of G418-resistant embryonic stem clones were digested with *Bam*HI and analyzed by Southern blot analysis using a *Bam*HI–*Eco*RV 2.5 kb external probe which recognizes a region upstream of the B3 fragment (Fig. 1A). This probe detects a 9 kb *Bam*HI fragment of the wild-type allele and a 7 kb fragment of the disrupted allele. One of the embryonic stem cell clones, I-20, contained a copy of the disrupted c-Rel locus and was injected into pseudopregnant female C57/Bl6 mice in the transgenic/knockout mice facility at the Rockefeller University. Twenty-four chimeric mice were obtained displaying 85–95% chimerism. Of these, 14 of the 19 male chimeras had germline transmission of the c-Rel gene-targeted allele. Heterozygotic F_1 mice were obtained through the breeding of male chimeras with C57BL/6 females. Southern blot analysis and PCR were used for subsequent genotyping.

Western blot analysis

Splenic lysates were prepared with 1×RIPA buffer (16), resolved on SDS–PAGE, transferred to nitrocellulose filter and subjected to Western blot analysis (16). Equal amounts of protein (40 μg) were loaded in each lane as was confirmed by Ponceau S staining of the membrane. The filter was first probed with the anti-c-Rel antibody at 1:1000 dilution (or 100 ng/ml). Following the incubation with the secondary antibody (horseradish peroxidase conjugated anti-rabbit antibody), specific bands were visualized via the ECL kit according to the manufacturer's instruction (Amersham). The same filter was subsequently re-probed with anti-IκBα antibody, anti-p50 and anti-p65 antibodies as internal protein loading controls.

Flow cytometric analysis

Splenic single-cell suspensions (1×10^6 cells) were generated and stained with directly FITC- or phycoerythrin (PE)-conjugated antibodies for 30 min at 4°C in 1×HBSS (with 2% FCS, 0.1% sodium azide and 10 mM HEPES, pH 7.2), washed extensively and analyzed using a Coulter XL flow cytometry analyzer. Antibodies utilized for phenotypic characterization include: FITC–anti-CD3, FITC–anti-CD4, FITC–anti-CD8, FITC–anti-IL-2Rα and PE–anti-B220 (PharMingen, San Diego, CA). An Epics XL flow cytometer and XL software was used to collect data while WinMDI software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA) was used for analysis.

Analysis of cell proliferation

Partial purified T cells (80–85%) were obtained from crude spleen populations by passing through a nylon wool column to enrich for T cells. The T cells were further enriched (>95%) through antibody-complement-mediated lysis using HB38 (anti-MHC class II) and M3/84 (anti-Mac-3) hybridoma supernatants and Low-Tox M Rabbit complement (Cedarlane, Ontario Canada). Proliferation assays were performed by plating out 1×10^5 purified B or T cells in triplicate for each condition in 96-well plates. The concentrations of each stimulus for the proliferation and functional studies were: anti-CD3 (2C11), 1.0 μg/ml; phorbol myristate acetate (PMA), 10 μg/ml; ionomycin, 100 μg/ml; IL-2 (Endogen, Cambridge, MA), 125 pM. Prior to indicated time points, cultured cells were incubated with 1 μCi [^{3}H]thymidine for 6 h and incorporation into DNA quantified by scintillation.

ELISA

Splenic T cells were stimulated with 1.0 μg/ml of anti-CD3 (2C11) for indicated time points and culture supernatants were utilized for ELISA following the manufacturer's protocol (Endogen). For the expression of various cytokines (IL-2, IL-3 and GM-CSF), plates were coated with 100 μl of diluted coating antibodies directed against the specific cytokine overnight at room temperature followed by blocking with 200 μl of 10% FCS in PBS at room temperature for 3 h. After extensive washing with 50 mM Tris (pH 7.0) plus 0.2% Tween 20, lymphocyte supernatant was added to each well in serial dilutions in triplicate and incubated overnight at 4°C. The plates were then incubated with the biotinylated detecting antibody (100 μl/well) and incubated for 1 h at room temperature. After extensive washing,

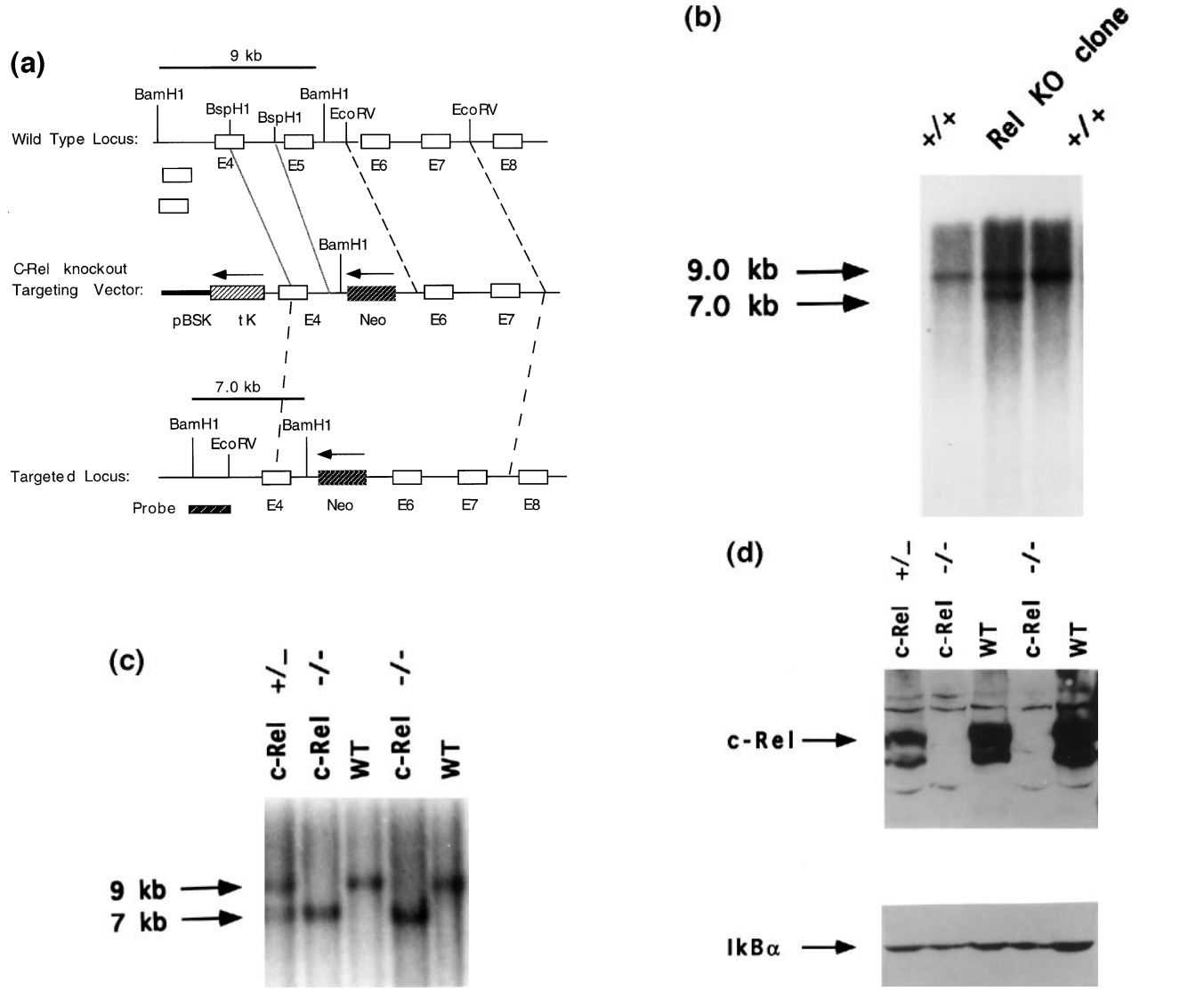

Fig. 1. Generation of the c-Rel knockout mice. (A). The c-Rel locus and the targeting-deletion c-Rel construct. The structure of the c-Rel wild-type genomic DNA, the knockout targeting construct and the targeted locus is shown. Exons are represented by open boxes and numbered. Exons 4 and 5 encode amino acids 51–101 and 102–130 of the c-Rel protein respectively, which are located within the Rel-homologous domain. Homologous recombination between genomic and the deletional construct is expected to result in a deletion of exon 5 of the c-Rel gene and a replacement with the *neo* gene. The length of the diagnostic *Bam*HI restriction fragments using the external probe (*Bam*HI–*Eco*RV 2.5 kb fragment) in Southern blot analysis is 9.0 kb for the wild-type locus and 7.0 kb for the gene-targeted c-Rel locus (B). Southern blot analysis of genomic DNA of the embryonic stem cell clones. Embryonic stem cell line, J1, was transfected with the c-Rel knockout targeting vector described in (A) by electroporation. Several hundreds of G418- and ganciclovir-resistant clones were analyzed by Southern blot analysis, following *Bam*HI digestion, for homologous recombination at the c-Rel locus using the *Bam*HI–*Eco*RV 2.5 kb probe. A representative clone with the targeted deletion at one c-Rel allele as indicated by the presence of the 7.0 kb *Bam*HI fragment is shown in lane 2. The wild-type allele generated the 9.0 kb *Bam*HI fragment. (C) Southern blot analysis of genomic DNA of the F_2 mice derived from inter-breeding of the heterozygous c-Rel-deficient F_1 mice (c-Rel$^{+/-}$). Genomic DNAs were digested with *Bam*HI and hybridized with the *Bam*HI–*Eco*RV 2.5 kb probe as described in (A). Results from five representative F_2 mice are shown. c-Rel$^{+/-}$, heterozygote; c-Rel$^{-/-}$, homozygote; WT, wild-type. As depicted, the mouse represented in lane 1 (c-Rel$^{+/-}$) is a heterozygote as it contains both the wild-type 9 kb and the deleted 7 kb bands. The mice represented in lanes 2 and 4 (c-Rel$^{-/-}$) are homozygous c-Rel knockout mice as they contain only the 7 kb restricted fragment. Lastly, DNA from wild-type mice in lanes 3 and 5 (WT) display only the 9 kb band. (D) Western blot analysis of splenic lysates derived from the c-Rel knockout $^{-/-}$, heterozygous ($^{+/-}$) and wild-type ($^{+/+}$) F_2 mice. Total spleen lysates obtained from the same F_2 mice analyzed in (C) were separated by SDS–PAGE, transferred onto nitrocellulose membrane and blotted with the anti-c-Rel-specific antibody (upper panel). The bottom panel shows the same filter that was subsequently re-probed with the anti-IκBα antibody to serve as protein loading control. Note that no c-Rel protein was detected in the c-Rel$^{-/-}$ lysates, indicating a gene-targeted disruption of the c-Rel locus as expected.

visualization of the reaction results was achieved by incubation with horseradish peroxidase-conjugated streptavidin and development of the colorimetric reaction in the presence of appropriate substrate, tetramethylbenzidine. For all cytokines tested, standard curve with ranges of 1.0–65 pM (IL-2), 0.4–13 pM (IL-3) and 1.5–85 pM (GM-CSF)

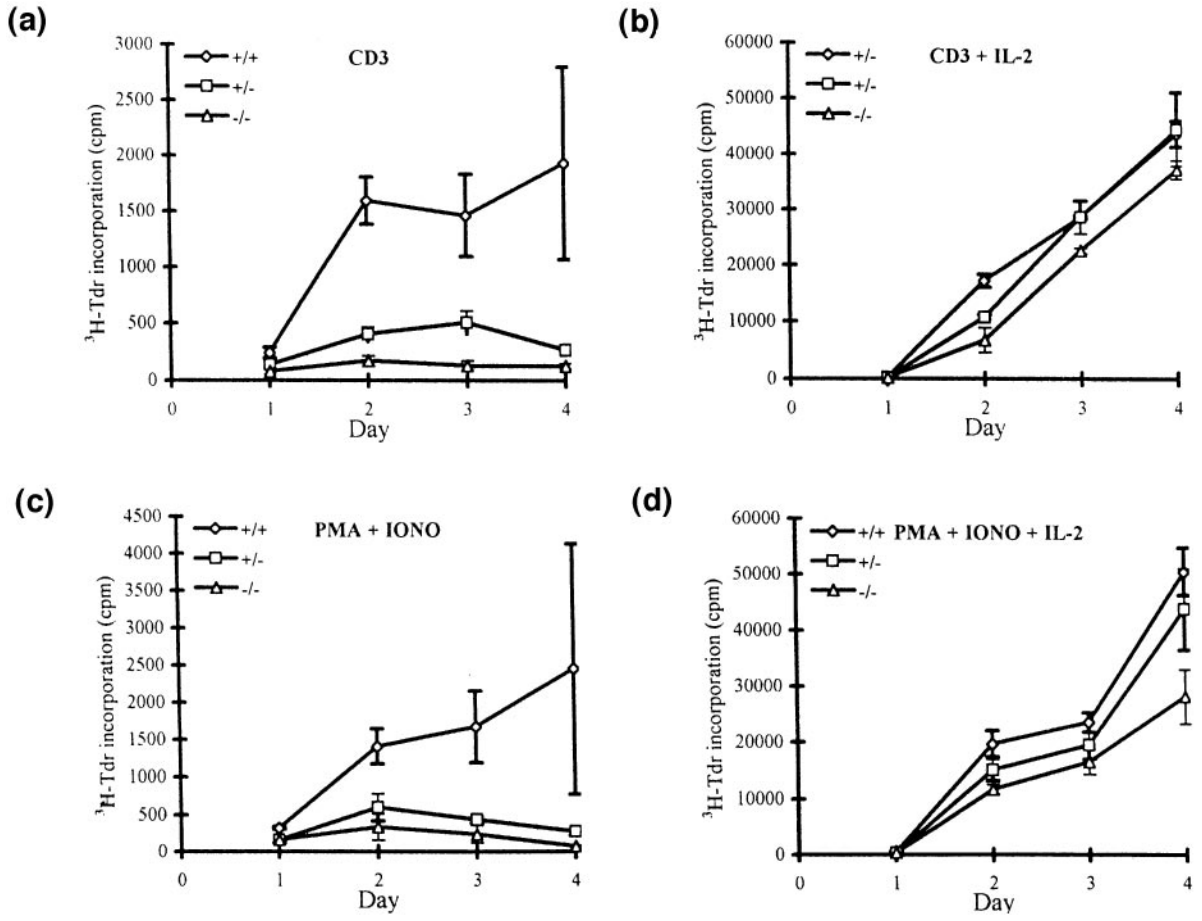

Fig. 2. c-Rel-deficient T cells fail to proliferate in response to various stimuli. Purified T cells were stimulated with 1 μg/ml of anti-CD3 (A and B) or PMA/ionomycin (10 and 100 μg/ml) respectively (C and D). Recombinant IL-2 (125 μM) was added in experimental groups in (B) and (D). Each day, one of the four replicate plates was assayed for [^{3}H]thymidine incorporation as a measure of cell proliferation. Each data point represents triplicate samples. For the anti-CD3 experiment group, the T cells were co-cultured with irradiated syngeneic splenic cells as a source of co-stimulatory signals.

were determined using the recombinant cytokines provided in the ELISA Minikit.

Electrophoretic mobility shift assay (EMSA)

The nuclear and cytosolic extracts were prepared as the following (16). Purified T lymphocytes (10×10^6 cells per treatment) were harvested, washed once with PBS, resuspended into 100 μl of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT] plus 0.1% NP-40. Cells were lysed on ice for 2 min and checked for complete lysis under the microscope. Nuclei were spun down and the supernatant was saved as cytosolic fraction. The nuclear pellet was resuspended into 100 μl of Buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT], sonicated for 5 s to decrease viscosity and rotated in the cold room for 30 min. Ultracentrifugation was performed to remove insoluble debris. The supernatant represented nuclear extract.

The EMSA was performed as described previously (16). For antibody supershift and inhibition experiments, the p50 and p65 and control antibodies was purified by ammonium sulfate precipitation of crude immune rabbit serum. C-Rel, p52 and RelB antibodies were commercially available (Santa Cruz Biotechnology, Santa Cruz, CA). The optimal amount of antibodies used in EMSA inhibition was pre-determined using purified proteins. For a 20 μl DNA binding reaction, 0.1 μg of purified antibody was incubated with 10 μg of nuclear extract and 1 μg of poly(dI–dC) in 1×DNA binding buffer on ice for 30 min before the addition of ^{32}P-labeled IgκB probe. The reaction was incubated at room temperature for 15 min and loaded onto a 6% native polyacrylamide gel. For high-resolution electrophoresis, gels were pre-run at 160 V for 2.5 h and continued for 3–3.5 h after the samples were loaded.

Generation of cytotoxic T lymphocytes (CTL) and T_h clones

To generate the CTL cell lines, we took 5×10^6 whole splenocytes from either wild-type, heterozygous or c-Rel null mice and co-cultured them in final media (RPMI 1640, 10% FCS, 50 μM β_2-mercaptoethanol) with equivalent numbers of γ-irradiated whole BALB/c splenocytes in the presence of recombinant IL-2 (125 pM) for 10 days. Cells were then harvested, and residual $CD4^+$ cells depleted using an anti-

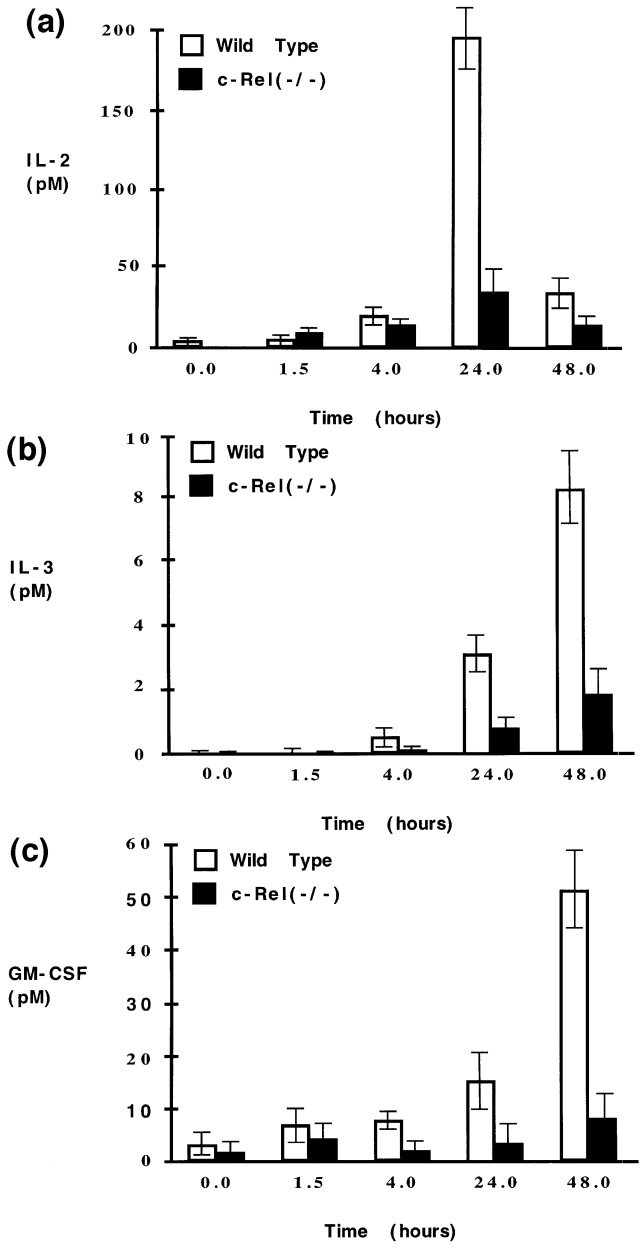

Fig. 3. Impaired cytokine production in c-Rel-deficient splenocytes stimulated with anti-CD3 *in vitro*. For *in vitro* stimulation, splenocytes from wild-type or c-Rel$^{-/-}$ mice were unstimulated or stimulated with 1 μg/ml of anti-CD3 for indicated time periods. Tissue culture supernatants, in triplicates, were subsequently assayed for the production of IL-2 (A), IL-3 (B) and GM-CSF (C), by ELISA following the manufacturer's protocol (Endogen, Cambridge, MA). Recombinant cytokines provided in the ELISA Minikit were utilized to generate standard curves with ranges of 1.0–65 pM (IL-2), 0.4–13 pM (IL-3) and 1.5–85 pM (GM-CSF) for quantification of the test supernatants.

L3T4 mAb (GK1.5) and anti-mouse IgG magnetic beads (Perceptive Diagnostics, Cambridge MA).

To generate the T_h cell line, we took 5×10^6 splenocytes from either wild-type or c-Rel null mice and cultured in final media in the presence of 1 μg/ml staphyloccocal enterotoxin B (SEB) (Toxin Technologies, Madison, WI) and 125 pM IL-2. After 10 days, residual CD8$^+$ cells were depleted by treatment with an anti-LY2 mAb (TIB 105) and anti-mouse IgG magnetic beads. Cells were subsequently re-stimulated and expanded in the presence of IL-2.

Measurement of cytotoxicity and helper functions of T cell clones

Cytotoxicity assays were performed as follows. Target cells bearing either the allogeneic haplotype (P815; MHC d haplotype) or autologous haplotype (EL-4; MHC b haplotype) were labeled for 1 h in 100 μl of 1 μCi/ml $Na^{51}Cr$ (Amersham). Cells were then extensively washed and 5×10^3 cells cultured per well in triplicate in the presence of increasing numbers of the CTL effector clones. After 4 h of incubation at 37°C, cells were pelleted and 100 μl of supernatant recovered for scintillation counting. Percent specific lysis was calculated as the percent c.p.m. of maximum (1% Triton-X control) obtained per sample after subtracting away the minimum control (media only). Percent lysis = 100×(sample release – spontaneous release)/(maximum release – spontaneous release).

T_h cell assays were performed as follows. Splenic B cells were purified from wild-type mice by mAb and complement-mediated lysis of T cells with a concurrent adherence step to deplete macrophages. Briefly, splenocytes were treated with mAb directed against Thy1 (J1J), L3T4 (GK1.5) and Ly2 (TIB 105), and subjected to two rounds of Low Tox M complement (Cedarlane). Cells were verified by flow cytometry to be >98% B220$^+$. Responder B cells (1×10^5) were then co-cultured in triplicate, alone or with 1×10^4 γ-irradiated T_h cells, in the presence or absence of 1 μg/ml SEB. At the times indicated, cultures were pulsed with 1 μCi [^{3}H]thymidine for 6 h before termination of culture. Cultures were harvested onto glass fiber filters and incorporation measured by liquid scintillation.

Results

Phenotypic characterization of the c-Rel knockout mice

To generate mice deficient for c-Rel, a genomic DNA fragment containing exon 5, which encodes amino acids 102–130 of the highly conserved Rel homologous domain, was targeted for deletion and replaced by an insertion of the neomycin resistance gene (Fig. 1A). The c-Rel deletional gene-targeting construct (Fig. 1A) was transfected into the embryonic stem cell line, J1 (29). One of the c-Rel-knockout embryonic stem cell clones, I-20, was found to contain a copy of the disrupted c-Rel locus (Fig. 1B) and was subsequently injected into pseudopregnant female C57BL/6 mice. Through breeding of these chimeras with C57BL/6 females, 10 out of the 14 male chimeras generated had germline transmission of the disrupted c-Rel gene as assessed by the transmission of the targeted allele to the F_1 offspring. Further breeding of the heterozygous F_1 mice generated c-Rel homozygous knockout mice in F_2 (Fig. 1C). All genotypic analyses were performed by Southern blot analysis of the tail-tip DNAs from 4- to 5-week-old mice using a diagnostic external probe, the 2.5 kb *Bam*HI–*Eco*RV fragment, located N-terminal to the targeted region (Fig. 1A).

The spleen cell lysates extracted from five representative

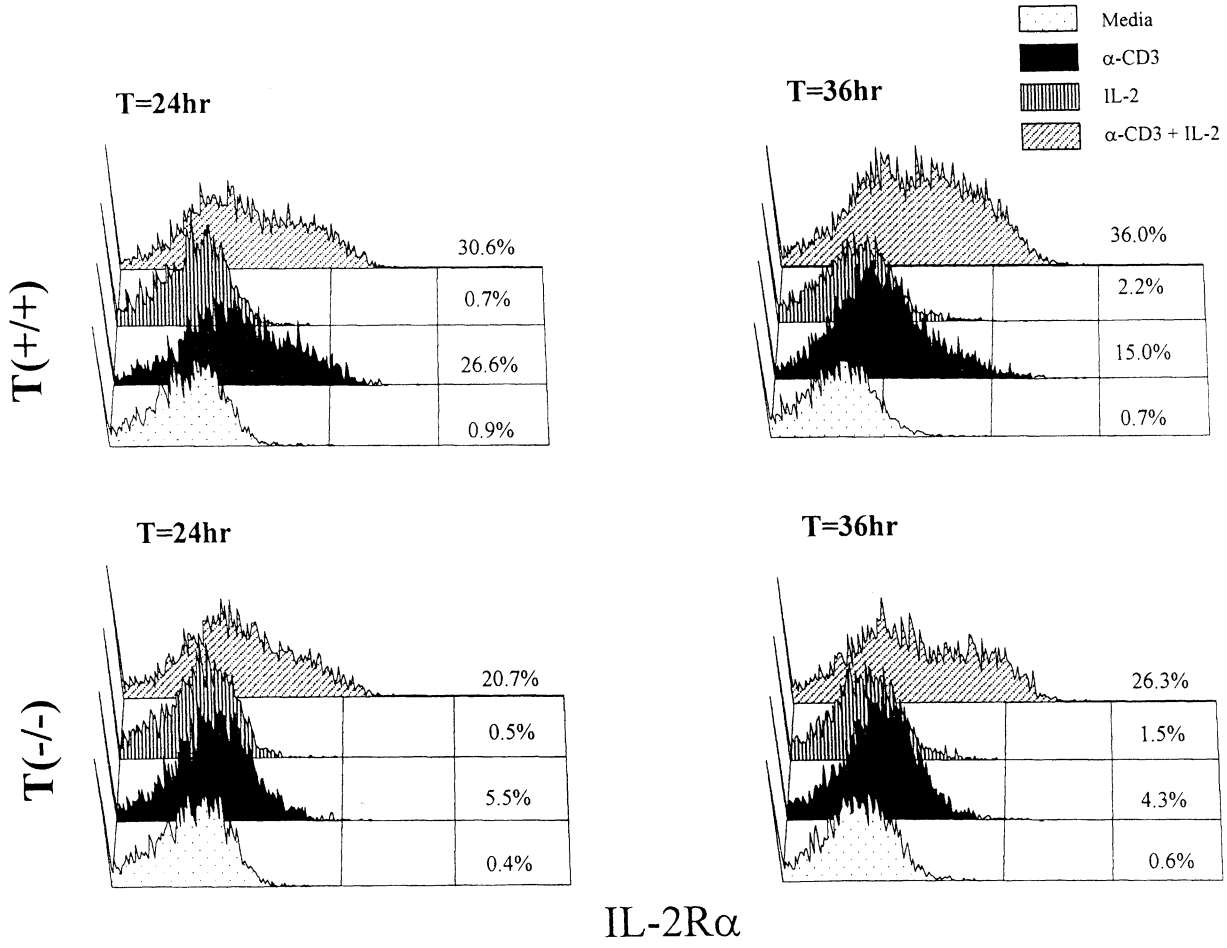

Fig. 4. IL-2Rα induction is impaired in c-Rel-deficient T lymphocytes. Wild-type or c-Rel$^{-/-}$ T cells were stimulated with either anti-CD3 (1 μg/ml) or IL-2 (125 μM), alone or in combination. At the indicated time points, CD25 (IL-2Rα) surface expression was assessed by flow cytometry by gating exclusively on viable cells. The percentage of positive cells and mean channel fluorescence (parenthesis) are represented numerically to the right of the histogram. C-Rel$^{-/-}$ T cells demonstrate impaired induction of IL-2Rα expression upon antigen-receptor stimulation. This defect is corrected upon addition of exogenous IL-2 in c-Rel$^{-/-}$ T cells.

mice were subjected to Western blot analysis using a polyclonal anti-c-Rel antibody. While the splenic lysates from two representative wild-type mice express high levels of the c-Rel protein, the lysates obtained from c-Rel homozygous knockouts have no expression of the c-Rel protein (Fig. 1D), confirming a successful deletion in the c-Rel gene locus. Interestingly, a gene-dosage effect was observed, in that the c-Rel heterozygote lysates contain about half the amount of the c-Rel protein as do wild-type lysates. As a protein loading control, the same filter was subsequently reprobed with antibodies recognizing other proteins, including IκBα, p50 and p65. The expression levels of IκBα, p50 and p65 are comparable in all lysates (Fig. 1D, bottom panel; data not shown), suggesting that: (i) the loss of c-Rel had no demonstrable effect on these family members and (ii) the proteins were equally loaded in all samples analyzed. Taken together, these experiments indicate that we have successfully created c-Rel knockout mice which do not express the c-Rel protein.

The c-Rel$^{-/-}$ mice are fertile and appeared overtly normal at a Mendelian ratio when housed in a pathogen-free facility. Phenotypic characterization of the lymphoid tissues demonstrated a normal development of T and B cell compartments. In particular, c-Rel knockout mice have normal sized thymi and spleens. Flow cytometric analysis of the c-Rel knockout thymus indicated a normal distribution of CD4 and CD8 single-positive T cells, as well as double-positive and double-negative T cell populations when compared to the wild-type mice (data not shown). In cells from spleens and lymph nodes, the number and ratio of B and T cells were also comparable to the normal mice (data not shown). These analyses indicated that B and T lymphocyte development is not apparently disturbed in the absence of c-Rel protein.

Activation of proliferation in c-Rel deficient T lymphocytes

Since members of the NF-κB/Rel family are activated by TCR signals, we investigated whether the disruption of the c-Rel gene affected TCR-mediated activation of proliferation. Consistent with previous observations (13), we found that T cells purified from the c-Rel$^{-/-}$ mice had defective proliferative responses to both anti-CD3, as well as PMA/ionomycin stimu-

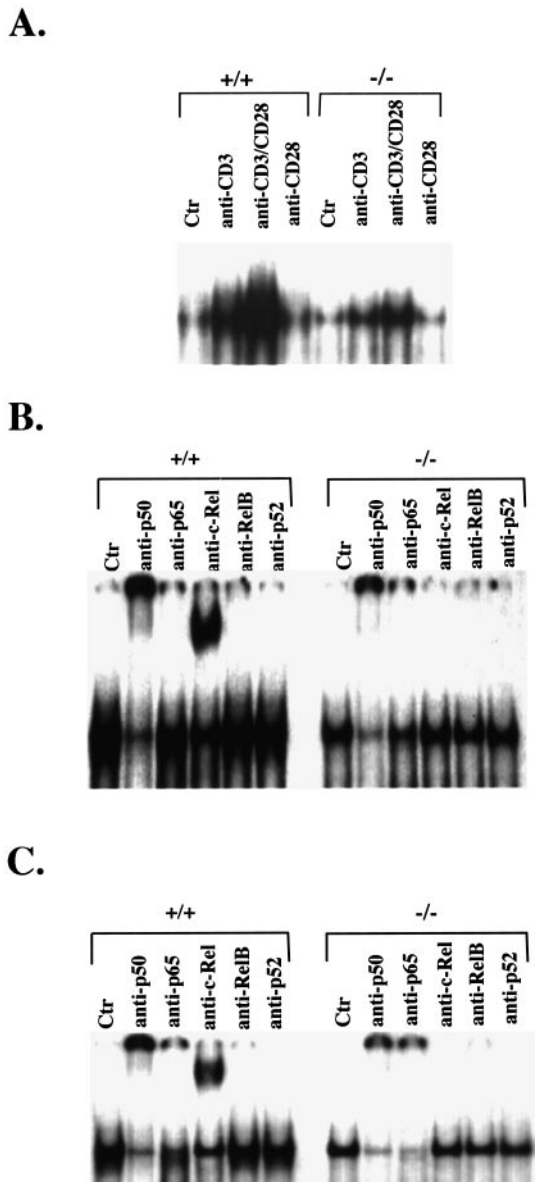

Fig. 5. EMSA of NF-κB/Rel nuclear translocation in c-Rel-deficient T lymphocytes. (A) Nuclear extracts of purified wild-type or c-Rel$^{-/-}$ T cells stimulated with indicated agonist antibodies for 6 h were prepared and incubated with ^{32}P-labeled IgκB oligonucleotide and analyzed by EMSA on 6% non-denaturing gel. Ten micrograms of each sample was loaded on individual lanes. Both anti-CD3 and anti-CD3 + anti-CD28 treatments induced 3- and 8-fold increase of NF-κB/Rel complexes over the unstimulated control as quantified by phosphor imager analysis. The fold induction in c-Rel$^{-/-}$ B cells stimulated with anti-CD3 or anti-CD3 + anti-CD28 is 1.5- and 3-fold over the control respectively. (B and C) NF-κB/Rel specific antibodies were utilized in the DNA binding reaction to identify the components in the NF-κB/Rel complexes. In the anti-CD3 (B) or anti-CD3 + anti-CD28 (C) stimulated wild-type T cell nuclear extract, the NF-κB/Rel complexes were supershifted or inhibited by antibodies against p50, p65 and c-Rel. In the c-Rel$^{-/-}$ cells, the binding complexes were inhibited by antibodies against p50 and p65 only, as c-Rel gene has been disrupted. Autoradiographs were exposed on X-ray films for 3 days for (B) and 1 day for (C).

lation, as compared to the wild-type cells (Fig. 2). The response of c-Rel heterozygous T cells were intermediate between the wild-type and the homozygous c-Rel knockout T cells, thus demonstrating a clear gene dosage effect. By comparison, there was no defect in proliferative responses of c-Rel knockout T cells, provided IL-2 was supplied exogenously (Fig. 2B and 2D).

Impaired cytokine production in c-Rel deficient T lymphocytes

T cell activation through the TCR and co-stimulatory molecules results in the production of cytokines. Therefore, the proliferative defects in c-Rel$^{-/-}$ T cells could be explained simply by impaired cytokine production, as NF-κB/Rel binding sites are present in the promoter regions of several cytokines and cytokine receptors, including IL-2, IL-3, GM-CSF, TNF-α, IL-6, and IL-2Rα chain (6,12,27,30–33). To focus specifically on the contribution of c-Rel in cytokine production, we took culture supernatants from wild-type or knockout splenocytes cultured with or without anti-CD3 antibody in the presence of irradiated antigen-presenting cells and analyzed for cytokine production by ELISA (Fig. 3A–C). Wild-type splenocytes treated with anti-CD3 produced detectable levels of IL-2, IL-3 and GM-CSF, although with varied kinetics. In cultures of wild-type cells, IL-2 levels peaked at 24 h (~186 pM) and declined by 48 h (~33 pM); whereas IL-3 and GM-CSF levels became detectable only after 48 h of stimulation (~8 pM for IL-3; ~54 pM for GM-CSF). By comparison to wild-type, splenocytes from c-Rel knockout mice produced <25% normal amounts of IL-2 (~30 pM), IL-3 (~2 pM) and GM-CSF (~8 pM).

Impaired IL-2Rα expression in c-Rel-deficient T lymphocytes

Since the IL-2Rα promoter contains an NF-κB/Rel regulatory element (31,32,34), we chose to follow the cell surface expression of the IL-2Rα chain on wild-type and c-Rel$^{-/-}$ T cells. As expected, anti-CD3 ligation of wild-type T cells induced 26.6% of the cells to become IL-2Rα^{+} by 24 h (Fig. 4, top two panels), and by 36 h, this expression level had decreased to 15.0%. In contrast to wild-type cells, only 5.5% of c-Rel$^{-/-}$ T cells became IL-2Rα^{+} by 24 h, with little change was observed at 36 h (Fig. 4, bottom two panels).

IL-2 up-regulates IL-2Rα by activating STAT5-responsive elements in the IL-2Rα promoter (32). Therefore, we examined the effect of adding exogenous IL-2 to our culture system, particularly as c-Rel$^{-/-}$ cells were deficient in IL-2 production. The addition of 125 pM IL-2 resulted in a slight up-regulation of IL-2Rα expression by wild-type cells (30% at 24 h and 36% at 36 h), but enhanced expression by the c-Rel$^{-/-}$ T cells was even more noticeable (20% at 24 h and 26% at 36 h) when stimulated with anti-CD3 antibody (Fig. 4). Consistent with the lack of functional IL-2Rα expression by resting T cells, IL-2 alone had no effect on IL-2Rα expression. Therefore, these data indicated that c-Rel$^{-/-}$ T cells have an impaired proliferative response to TCR signals as a consequence of the decreased production of IL-2 and IL-2Rα, but supplement with exogenous IL-2 circumvents such deficiency.

Reduced nuclear translocation of NF-κB/Rel components

To determine whether the decreased cytokine and receptor production results from a diminution in the NF-κB/Rel nuclear

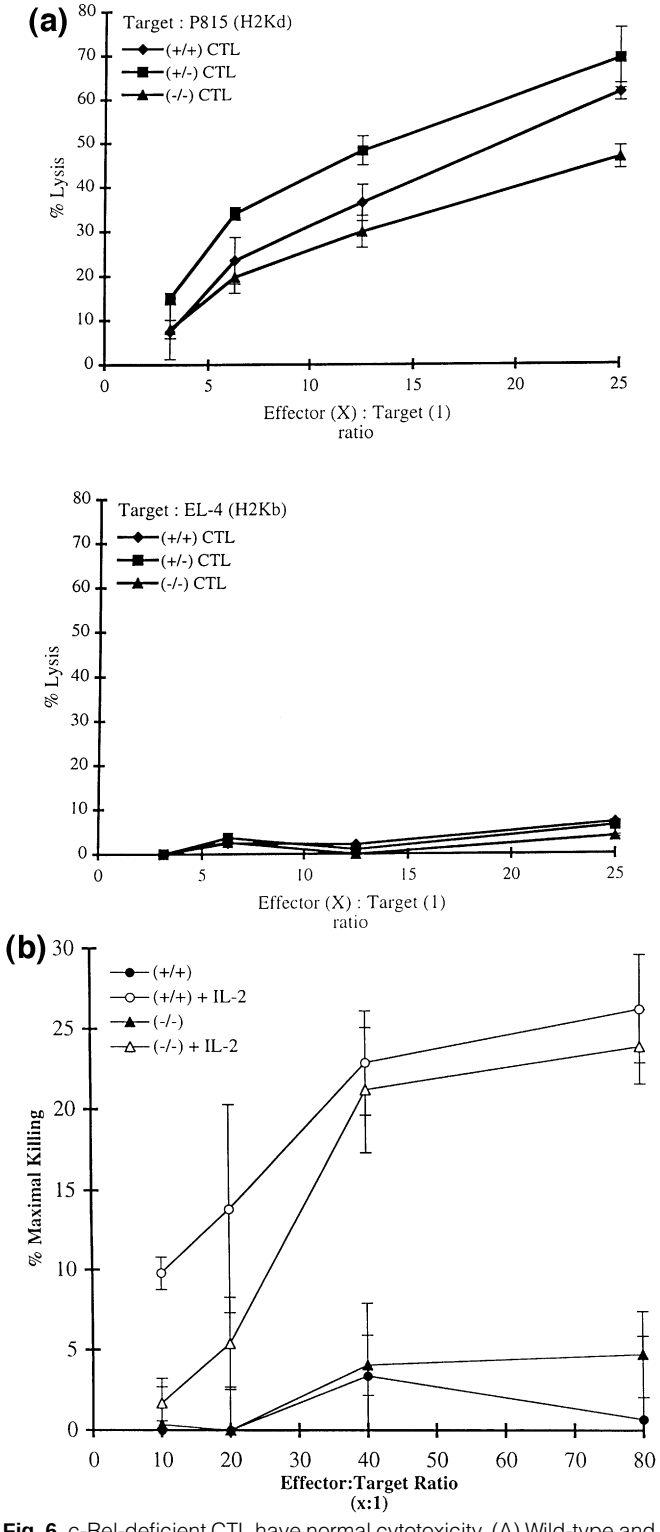

Fig. 6. c-Rel-deficient CTL have normal cytotoxicity. (A) Wild-type and c-Rel$^{-/-}$ CTL lines were generated against irradiated BALB/c splenocytes (H2-K^d) as stimulators in the presence of exogenous IL-2. ^{51}Cr-labeled H2-K^d-bearing (P815) or H2-K^b-bearing (EL-4) target cells were co-cultured with either wild-type or c-Rel$^{-/-}$ CTL lines, and cytotoxicity was assayed at the indicated effector (CTL):target ratios. After 4 h of co-culture, supernatants were collected and specific cell lysis assessed by scintillation counting. (B) Cytotoxicity assay was performed using freshly purified naive wild-type and c-Rel$^{-/-}$ CD8$^+$ T cells.

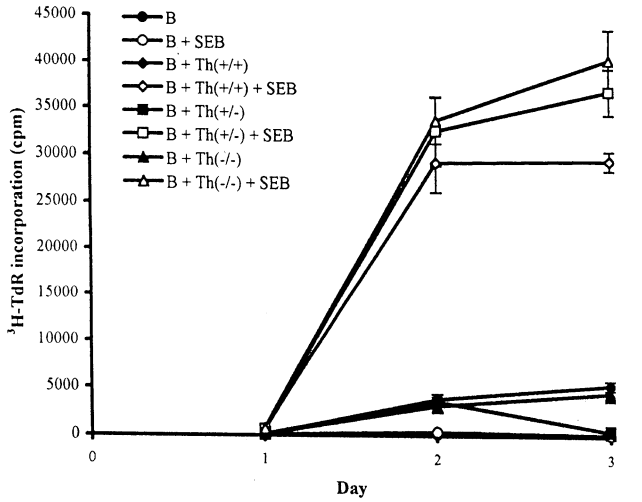

Fig. 7. CD4$^+$ T$_h$ cells derived from the c-Rel knockout mice provide help to B cells. Purified wild-type responder B cells (1×10^5) were co-cultured in triplicate alone or with 1×10^4 γ-irradiated T$_h$ cells from wild-type (+/+), c-Rel heterozygotes (+/–) or c-Rel knockout homozygotes (–/–) mice in the presence or absence of 1 μg/ml SEB in 96-well plates. At the times indicated, cultures were pulsed with 1 μCi [^{3}H]thymidine. After 6 h of incubation, thymidine incorporation was measured by liquid scintillation.

complexes, we performed EMSA on nuclear extracts prepared from stimulated T cells. Ligation of the TCR receptor alone, or in conjunction with CD28 co-stimulation, caused a 3- and 8-fold induction of nuclear translocation of NF-κB/Rel binding complexes in wild-type T cells (Fig. 5A). By comparison, the same stimulation of c-Rel$^{-/-}$ T cells, caused only a modest induction of NF-κB/Rel binding complexes, with a 1.5- to 3-fold increase over the unstimulated control.

The composition of the NF-κB/Rel complexes was assessed by using antibodies in which family member specificity was verified and optimized by reactions with purified NF-κB/Rel proteins (16). The results demonstrated that the NF-κB/Rel complexes in the anti-CD3 or anti-CD3 + anti-CD28 stimulated wild-type nuclear extracts were supershifted or inhibited by anti-p50, anti-p65 and anti-c-Rel antibodies, but unaffected, or affected only slightly by anti-RelB or anti-p52 antibodies (Fig. 5B and C). As expected, in c-Rel$^{-/-}$ nuclear extracts the NF-κB/Rel complexes were supershifted only by the anti-p50 and anti-p65 antibodies, but not by anti-c-Rel antibody. The EMSA results implicate that, in the c-Rel deficient T cells, there was a decreased NF-κB nuclear translocation upon TCR or TCR/CD28 stimulation, which correlates with the impaired cytokine and receptor gene expression.

Cytotoxicity of c-Rel-deficient CD8$^+$ T cells

T lymphocytes require TCR and co-stimulation signals in order to differentiate into CD8$^+$ effector cytotoxic or CD4$^+$ T$_h$ cells (35–38). Since c-Rel$^{-/-}$ T cells are deficient in cytokine production in the initial activation phase, they may be unable to differentiate into functional effector cells (38). However, since IL-2 can restore the proliferation defects, we tested whether c-Rel is required for effector function by using IL-2 supplement medium to bypass the activation requirement for T cell proliferation and differentiation.

To assess the role of c-Rel in CTL function, we generated allo-specific $CD8^+$ T cells by co-culturing either wild-type or c-Rel null whole splenocytes ($H\text{-}2^b$) with irradiated BALB/c ($H\text{-}2^d$) splenocytes in the presence of 125 pM IL-2. After 10 days of culture and an enrichment step for $CD8^+$ (to >95%), cells were assayed for their ability to lyse either MHC-matched or mismatched haplotypic targets, EL-4 ($H\text{-}2^b$) or P815 ($H\text{-}2^d$) respectively. As shown in Fig. 6(A), the wild-type $CD8^+$ T cells lysed the allotypic-target cells P815 effectively, but not the MHC-matched EL-4 cells, as expected. The c-$Rel^{-/-}$ $CD8^+$ cells had slightly reduced cytolytic capacity. However, the target cell specificity was comparable to that of wild-type cells in that they only lysed the target cells, P815, with allotypic MHC. Thus, in the presence of IL-2, c-$Rel^{-/-}$ CD8 cells are capable of differentiating into fully functional cytotoxic T cells, suggesting that c-Rel plays little role in cytotoxicity effector function. Since cytotoxicity requires preactivation of lymphocytes, we could not detect cytotoxicity in freshly purified $CD8^+$ cells of either wild-type or c-$Rel^{-/-}$ origins without IL-2 pre-activation (Fig. 6B).

Helper function of c-Rel deficient $CD4^+$ T cells

It has been well-documented that T–B collaboration provides an important mechanism for T cells to provide help to enhance B cell proliferation and antibody production. The key surface molecules involved in T–B collaboration are TCR/CD28/CD40L on T cells and MHC/B7/CD40 on B cells (39–42). Since T–B collaboration involves multiple surface ligand–receptor interactions, it was of interest to determine whether c-Rel influenced, in any way, the differentiation of $CD4^+$ T cells into fully functional T_h lymphocytes. Because antigen-specific T_h cells are found at a much lower frequency than allo-reactive T cells, we utilized the phenomenon of superantigen-mediated cognate T–B interaction (36–38) to assess whether SEB-reactive T_h cells derived from either the wild-type or a c-Rel knockout mice were capable of inducing B cell proliferation. Since we knew that c-$Rel^{-/-}$ T cell clone could not be generated in the absence of IL-2, we cultured splenocytes of both wild-type and c-$Rel^{-/-}$ mice in the presence of SEB plus exogenous IL-2. After several days of culture and a subsequent enrichment for $CD4^+$ (to >95%), cells were allowed to quiescence upon the withdrawal of IL-2 for 48 h before harvested for γ-irradiation treatment. Normal splenic B cells were then purified and co-cultured with the irradiated T_h cells in the presence or absence of SEB. In this setting, functional T_h cells drive B cell proliferation only in the presence of the appropriate superantigen (36–38). Our studies indicated that both wild-type and c-Rel knockout $CD4^+$ lymphocytes are fully capable of inducing wild-type B cell proliferation in the presence of the superantigen (Fig. 7). This activity was normal in the regard that the amplitude and the specificity of the response induced by the knockout cells was comparable to that of wild-type. In supporting this observation, the expression level of CD28 and CD40L on c-$Rel^{-/-}$ T cells treated with anti-CD3 is comparable to that of wild-type T cells (data not shown). Taken together, our studies on the CTL and T_h function indicated that c-Rel is crucial for the proliferation and differentiation into these effector cells. However, once the proliferation is overcome by the addition of exogenous IL-2, c-$Rel^{-/-}$ T cells can fully execute cytotoxicity or helper function.

Discussion

The results of this investigation indicate that c-Rel is the predominant member of the NF-κB/Rel family of transcription factors that are involved in the promotion of IL-2, IL-3, and GM-CSF and IL-2Rα expression. A deficiency of c-Rel is not compensated for by the other two members of the NF-κB/Rel family, p50 and p65, which are also activated by the TCR and accessory molecules. Our studies further demonstrated that once the activation block is corrected with the addition of IL-2, c-Rel-deficient T cells can differentiate into functional cytotoxic T cells and T_h, suggesting its specific window of action at the immediate early phase of T cell activation.

c-Rel is essential for IL-2 and IL-2Rα expression

The profound deficiency of expression of the IL-2 gene by cells from c-$Rel^{-/-}$ mice was unanticipated, given the established relative importance of NF-AT in the activation of IL-2 gene expression. However, the importance of NF-κB/Rel in the activation of IL-2 gene expression could have been predicted, given the profound effect of glucocorticoids on IL-2 production. Recent reports have now shown quite convincingly that glucocorticoids inhibit cytokine gene expression by inducing the expression of IκB, which then prevents NF-κB/Rel activation by sequestering NF-κB/Rel factors in the cytoplasm (43,44). In addition, activated glucocorticoid receptors can prevent NF-κB/Rel transcriptional activity by interacting directly with NF-κB/Rel proteins, prior to DNA binding (45).

Equally as unanticipated was the lack of an effect on IL-2 gene expression with disruption of either the NF-ATp or NF-ATc transcription factor. In particular, NF-ATp deficient T cells have dysregulated IL-4 production (46,47), whereas NF-ATc deficiency led to a decrease in IL-4 and IL-6 production (48,49). In both cases, NF-AT-deficient cells seem to have normal production of IL-2. These studies indicated that either disruption of these NF-AT members alone is not sufficient for interfering with IL-2 production or that other transcription factors, such as NF-κB/Rel, can compensate for its expression.

The *in vivo* footprinting studies performed by Dr Rothenberg and colleagues clearly indicated that individual IL-2 regulating transcription factors, NF-AT, AP-1 and NF-κB/Rel, cannot stably bind their target sequences *in vivo* unless all the other factors are present at the neighboring sites (22,24). Recent X-ray crystal structure analysis demonstrated that the NF-AT, Fos and Jun, could form a stable tertiary complex which tightly associates with the composite NF-AT/AP-1 site in the IL-2 promoter (50). As the regulation of cytokine promoters is most likely coordinated by the combinatorial transcription factors, it should be acknowledged that NF-κB/Rel transcription factors may act synergistically with NF-AT and AP-1 in modulating the level of cytokine production in T cells (21,51–53). Therefore, it will now be interesting to extend this kind of structural analysis to include the NF-κB/Rel binding site and the NF-κB/Rel family members, to visualize the conformation of the entire 'enhansasome' bound to the IL-2 promoter.

It is intriguing to observe that the expression of the IL-2Rα chain is also dependent on the presence of c-Rel protein. The regulation of the IL-2Rα chain gene is distinct from the

IL-2 gene itself, in that IL-2Rα chain gene expression is controlled by both the TCR and IL-2 (54). Resting T cells express no detectable IL-2Rα chain, but within 3–6 h of stimulation via the TCR, the α chain first becomes detectable. Subsequently, the secretion of IL-2 results in a further activation of the IL-2Rα chain gene. The promoter region of IL-2Rα gene contains bona fide NF-κB/Rel and GAS sites; the latter interacts with the STAT5 transcription factor (55). The fact that IL-2 supplement, which induces STAT5 transcription factor, can fully restore the expression of IL-2Rα chain expression in the c-Rel$^{-/-}$ T cells suggest that both NF-κB/Rel and STAT5 transcription factors contribute to its expression, although in response to distinct signals: NF-κB/Rel binding site is crucial for the initial induction phase by TCR signal, whereas the STAT5 site would be important only after IL-2 becomes available (55).

c-Rel and T cell effector function

By taking advantage of superantigen-mediated T cell help, we were able to assess whether the c-Rel$^{-/-}$-derived CD4$^+$ T_h cells were capable of providing help to normal resting B cells. It needs to be emphasized that IL-2 was supplemented in culture medium in the generation of these T_h cell clones, as we will not be able to generate T cell clones in the absence of the proliferation cytokine. Yet, our studies indicated that once the activation and proliferation requirements are fulfilled, The T cell differentiation program can proceed without c-Rel protein. Superantigen-mediated interaction between T and B cells is in its nature mimicking the cognate interaction between T_h and B cells: both are engaged through interaction of TCR and MHC, as well as other co-stimulatory molecules, such as CD28/B7 and CD40 ligand–CD40 interaction. Indeed, we found that the expression levels of CD28 and CD40 ligand was normal in the c-Rel$^{-/-}$ T cells upon stimulation with anti-CD3 (data not shown), confirming the notion that these c-Rel-deficient T cells are capable of helping B cell proliferation

It was also surprising that disruption of the c-Rel locus seems to exert little effect on the ability of T lymphocytes to execute cytotoxicity function. It has been well-documented that the process that drives CTL precursors (CTLp) to the blastoid cytotoxic effector cells requires the signals from TCR and CD28 co-stimulation. In addition, the production of IL-2 and IL-2 receptors is crucial to the maturation and differentiation of CTL effectors, because the transformation of small non-granular CTL can be induced to large granular CTL by IL-2 (56). Similar to the approach described for the generation of T_h clones, we generated the CTL clones in the presence of IL-2 to circumvent the intrinsic proliferation defects of the c-Rel$^{-/-}$ T cells. Our studies indicated that once the activation phase was elicited, c-Rel$^{-/-}$ CTL has the full capacity to kill the allogeneic cell targets, suggesting the integrity of cytotoxicity machinery. This collective information thus implies that c-Rel is only required for the TCR-mediated blastoid transformation of CTLp and is not required for the second phase of maturation of CTL effectors triggered by IL-2.

In conclusion, c-Rel is critical for controlling the early activation events mediated by the antigen receptor signals in T lymphocytes, but it is dispensable for the execution of the effector function. Its effects, in the case of T lymphocytes, are mostly grossly manifested in proliferative defects resulting directly from a defined impairment in cytokine production and receptor expression. Future work will be necessary to explore the physiological function of the c-Rel gene in the host defense network against pathogenic infection.

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Abbreviations

EMSA	electrophoretic mobility shift assay
CTL	cytotoxic T lymphocyte
CTLp	precursor cytotoxic T lymphocyte
GM-CSF	granulocyte macrophage colony stimulating factor
PE	phycoerythrin
SEB	staphylococcal enterotoxin B
TNF	tumor necrosis factor

References

1 Sen, R. and Baltimore, D. 1986. Multiple nuclear factors interact with the immunoglobulin enhancers. *Cell* 46:705.
2 Baeuerle, P. A. and Baltimore, D. 1988. IκB: a specific inhibitor of the NF-κB transcription factor. *Science* 242:540.
3 Lenardo, M. J. and Baltimore, D. 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58:227.
4 Ghosh, S., *et al.* 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. *Cell* 62:1019.
5 Nolan, G. P., Ghosh, S., Liou, H.-C., Tempst, P. and Baltimore, D. 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. *Cell* 64:961.
6 Liou, H.-C. and Baltimore, D. 1993. Regulation of the NF-κB/Rel transcription factor and IκB inhibitor system. *Cur. Opin. Cell Biol.* 5:477.
7 Fujita, T., Nolan, G. P., Liou, H.-C., Scott, M. L. and Baltimore, D. 1993. The candidate proto-oncogene *bcl*-3 encodes a transcriptional co-activator that activates NF-κB p50 homodimers. *Genes Dev.* 7:1354.
8 Baeuerle, P. and Baltimore, D. 1996. NF-kB: ten years after. *Cell* 87:13.
9 Sha, W. C., Liou, H.-C., Tuomanen, E. I. and Baltimore, D. 1995. Targeted disruption of the p50 subunit of NF-kB leads to multifocal defects in immune responses. *Cell* 80:321.
10 Weih, F., *et al.* 1995. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kB/Rel family. *Cell* 80:331.
11 Burkly, L., *et al.* 1995. Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* 373:531.
12 Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S. and Baltimore, D. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kB. *Nature* 376:167.
13 Kontgen, F., *et al.* 1995. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev.* 9:1965.
14 Franzoso, G., *et al.* 1998. Mice deficient in Nuclear Factor (NF)-kB/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J. Exp. Med.* 187:147.

15 Caamano, J. H., *et al.* 1998. Nuclear Factor(NF)-kB2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med.* 187:185.
16 Liou, H.-C., Sha, W. C., Scott, M. L. and Baltimore, D. 1994. Sequential induction of NF-κB/Rel family proteins during B-cell terminal differentiation. *Mol. Cell Biol.* 14:5349.
17 Cantrell, D. A. and Smith, K. A. 1984. The interleukin-2 T-cell system: a new cell growth model. *Science* 224:1312.
18 Stern, J. B. and Smith, K. A. 1986. Interleukin-2 induction of T-cell G_1 progression and c-*myb* expression. *Science* 233:203.
19 Wang, W., Tam, W. F., Hughes, C. C., Rath, S. and Sen, R. 1997. c-Rel is a target of pentoxifylline-mediated inhibition of T lymphocyte activation. *Immunity* 6:165.
20 Northrop, J. P., *et al.* 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* 369:497.
21 Rooney, J. W., Sun, Y. L., Glimcher, L. H. and Hoey, T. 1995. Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. *Mol. Cell Biol.* 15:6299.
22 Chen, D. and Rothenberg, E. V. 1994. Interleukin 2 transcription factors as molecular targets of cAMP inhibition: delayed inhibition kinetics and combinatorial transcription roles. *J. Exp. Med.* 179:931.
23 Garrity, P. A., Chen, D., Rothenberg, E. V. and Wold, B. J. 1994. Interleukin-2 transcription is regulated in vivo at the level of coordinated binding of both constitutive and regulated factors. *Mol. Cell Biol.* 14:2159.
24 Rothenberg, E. V. and Ward, S. B. 1996. A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. *Proc. Natl Acad. Sci. USA* 93:9358.
25 Auphan, N., Di Donato, J. A., Rosette, C., Helmberg, A. and Karin, M. 1995. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 270:286.
26 Scheinman, R. I., Cogswell, P. C., Lofquist, A. K. and Baldwin, A. S., Jr. 1995. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* 270:283.
27 Gerondakis, S., *et al.* 1996. Rel-deficient T cells exhibit defects in production of interleukin 3 and granulocyte-macrophage colony-stimulating factor. *Proc. Natl Acad. Sci. USA* 93:3405.
28 Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T. and Mulligan, R. C. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-*abl* proto-oncogene. *Cell* 65:1153.
29 Li, E., Bestor, T. H. and Jaenisch, R. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69:915.
30 Grilli, M., Chiu, J.-S. and Lenardo, M. J. 1991. NF-κB and rel-participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.* 143:1.
31 Tan, T.-H., *et al.* 1992. κB-site dependent activation of the interleukin-2 receptor alpha-chain gene promoter by human c-rel. *Mol. Cell Biol.* 12:4067.
32 John, S., *et al.* 1995. Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. *Mol. Cell Biol.* 15:1786.
33 Weih, F., Warr, G., Yang, H. and Bravo, R. 1997. Multifocal defects in immune responses in RelB-deficient mice. *J. Immunol.* 158:5211.
34 Pierce, J. W., Jamieson, C. A., Ross, J. L. and Sen, R. 1995. Activation of IL-2 receptor alpha-chain gene by individual members of the rel oncogene family in association with serum response factor. *J. Immunol.* 155:1972.
35 DeFranco, A. L., Ashwell, J. D., Schwartz, R. H. and Paul, W. E. 1984. Polyclonal stimulation of resting B lymphocytes by antigen-activated T lymphocytes. *J. Exp. Med.* 159:861.
36 Tumang, J. R., Posnett, D. N., Cole, B. C., Crow, M. and Friedman, S. M. 1990. Helper T cell-dependent human B cell differentiation mediated by a mycoplasmal superantigen bridge. *J. Exp. Med.* 171:2153.
37 Tumang, J. R., *et al.* 1991. T helper cell-dependent microbial superantigen-induced murine B cell activation: polyclonal and antigen-specific antibody responses. *J. Immunol.* 147:432.
38 Henkart, P. A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity* 1:343.
39 Clark, L. B., Foy, T. M. and Noelle, R. J. 1996. CD40 and its ligand. *Adv. Immunol.* 63:43.
40 Noelle, R. J. 1996. CD40 and its ligand in host defense. *Immunity* 4:415.
41 June, C. H., Bluestone, J. A., Nadler, L. M. and Thompson, C. B. 1994. The B7 and CD28 receptor families. *Immunol. Today* 15:321.
42 Lenschow, D. J., Walunas, T. L. and Bluestone, J. A. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 14:233.
43 Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. and Karin, M. 1995. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. *Science* 270:286.
44 Scheinman, R. I., Cogsell, P. C., Lofquist, A. K. and Baldwin, A. S., Jr. 1995. Role of transcriptional activation of IkBa in mediation of immunosupression by glucocorticoids. *Science* 270:283.
45 Ray, A. and Prefontaine, K. E. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc. Natl Acad. Sci. USA* 91:752.
46 Hodge, M. R., *et al.* 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity* 4:397.
47 Xanthoudakis, S., *et al.* 1996. An enhanced immune response in mice lacking the transcription factor NF-AT1. *Science* 272:892.
48 Yoshida, H., *et al.* 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and T_h2 cytokine production. *Immunity* 8:115.
49 Ranger, A. M., *et al.* 1998. Delayed lymphoid repopulation with defects in Il-4-driven responses produced by inactivation of NF-ATc. *Immunity* 8:125.
50 Chen, L., Glover, J. N. M., Hogan, P. G., Rao, A. and Harrision, S. C. 1998. Structure of the DNA-binding domains from NF-AT, Fos, and Jun bound to DNA. *Nature* 392:42.
51 Lederer, J. A., Liou, J. S., Todd, M. D., Glimcher, L. H. and Lichtman, A. H. 1994. Regulation of cytokine gene expression in T helper cell subsets. *J. Immunol.* 152:77.
52 Timmerman, L. A., *et al.* 1997. Redundant expression but selective utilization of nuclear factor of activated T cells family members. *J. Immunol.* 159:2735.
53 Rooney, J. W., Hoey, T. and Glimcher, L. H. 1995. Coordinate and cooperative roles for NF-AT and AP-1 in the regulation of the murine IL-4 gene. *Immunity* 2:473.
54 Smith, K. A. and Cantrell, D. A. 1985. Interleukin 2 regulates its own receptors. *Proc. Natl Acad. Sci. USA* 82:864.
55 John, S., Robbins, C. M. and Leonard, W. J. 1996. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. *EMBO J.* 15:5627.
56 Berke, G. and Rosen, D. 1988. Highly lytic *in vivo* primed cytolytic T lymphocytes devoid of lytic granules and BLT-esterase activity acquire these constituents in the presence of T cell growth factors upon blast transformation *in vitro*. *J. Immunol.* 141:1429.