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The goal of this investigation is to determine whether modulation of NF-κB activity by genetic engineering of DCs can affect DC longevity and T cell stimulatory functions. The specific aims are: 1) To determine the effect of expression of constitutively-active IKKβ (IκB kinase) on nuclear NF-κB levels in DCs, and on the longevity of DCs. 2) To determine the effect of IKKβ expression on in vitro and in vivo T cell priming by DCs. Results from these studies may thus lead the way for development of approaches for enhancing DC survival and function, with the goal of generating potent anti-tumor immunity.

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Introduction

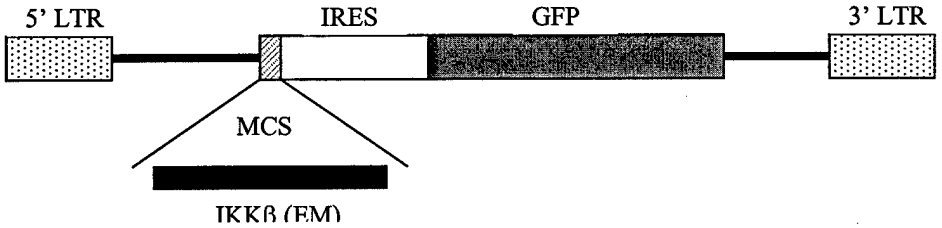
The goal of this investigation is to determine whether modulation of NF-κB activity by genetic engineering of dendritic cells (DCs0 can affect DC longevity and T cell stimulatory functions. The specific aims are: 1) To determine the effect of expression of constitutively-active IKKβ (IκB kinase) on nuclear NF-κB levels in DCs, and on the longevity of DCs. 2) To determine the effect of IKKβ expression on in vitro and in vivo T cell priming by DCs. Results from these studies may thus lead the way to development of approaches for enhancing DC survival and function, with the goal of generating potent anti-tumor immunity.

Body

I would first like to mention that unpublished data shown in the grant application were published last year in the journal *Immunity* (1). These published results further support a role for NF-kB proteins in regulating survival of DCs, which provide the basis for studies proposed in this application. Results pertaining to the goals of this application are described next.

We first generated an appropriate construct for performing the proposed studies. A constitutively-active mutant the NF-kB activating IKKβ kinase (CA-IKKβ) was kindly provided by the laboratory of Dr. Michael Karin (UC-San Diego)(2). CA-IKKβ was subcloned into the MIG retroviral vector (MIG-IKK)(Fig. 1). This vector also expresses the GFP protein using an IRES sequence to allow detection of infected cells. MIG-IKK was then transfected into the retroviral packaging line BOSC (3). Retroviral supernatants were collected 48hr and 72hr after transfection.

MIG:



pLL3.3-IRES-GFP:

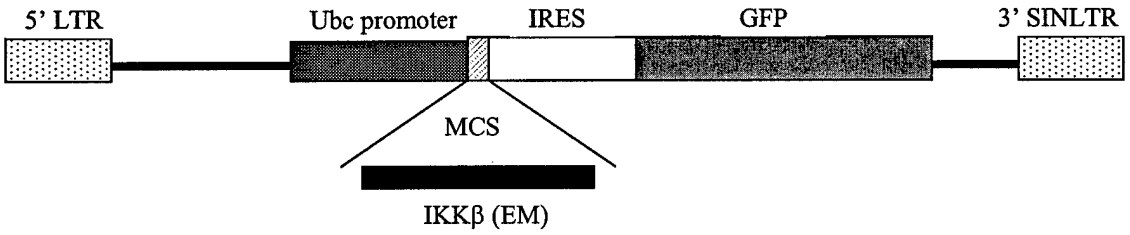


Fig. 1 The MIG retroviral vector (above) and lentiviral vector (below) described here.

We next determined whether infection with MIG-IKK virus was sufficient to activate NF-kB. To this end, we infected mouse fibroblasts (NIH-3T3) with MIG-IKK. Based on FACS analysis with GFP, greater than 90% cells were infected (i.e., GFP-positive) after 48hrs. We then determined NF-kB levels by EMSA, with and without TNFα treatment, in retrovirus infected cells. TNFα is a strong and well-characterized inducer of NF-kB and was used here to determine the efficacy of MIG-IKK expression-induced NF-kB activation. As shown in Fig. 2, untreated and uninfected cells have very little NF-kB binding activity, while TNFα can dramatically increase NF-kB levels after a 2hr treatment. Infection with the MIG control virus did not activate NF-kB, nor interfered with NF-kB activation by TNFα. Thus, retroviral infection of NIH-3T3 cells does not induce NF-kB activity in mouse fibroblasts. Strikingly, infection with MIG-IKK was sufficient to induce NF-kB in these cells to levels comparable to TNFα treatment. **This is an extremely important result for the studies proposed here, which are based on the effects of NF-kB activated by CA-IKKβ.**

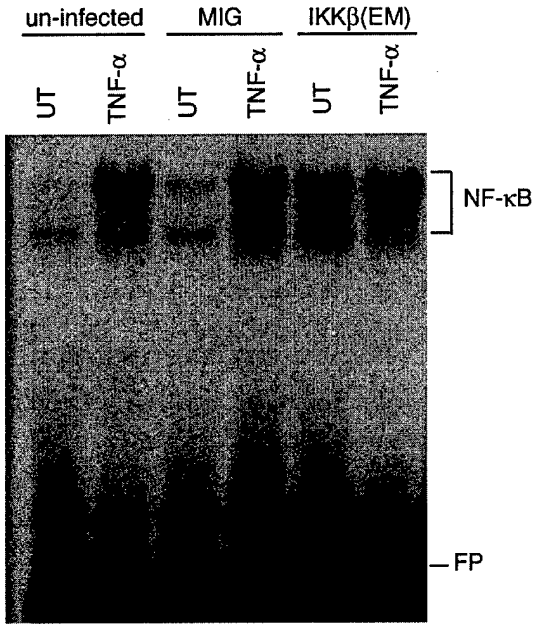


Fig. 2 NIH3T3 cells were uninfected/infected with MIG or MIG-IKK (IKKβ(EM)) retrovirus. 48 hours later, cells were treated with/without TNF-α (10 ng/ml) for 2 hours. Nuclear extracts were then made and subjected to EMSA.

Using the MIG control virus, we set about optimizing conditions for retroviral infection of mouse bone marrow-derived DCs. BM precursors were cultured in the presence of GM-CSF containing supernatant for 6 days. We tried many different procedures for optimal infection of DCs, but have found that infection on days 2 and 4 led to a high infection rate (73% GFP-positive DCs)(Fig. 3). We are now ready to study the effect of MIG-IKK infection on DC survival and other functions.

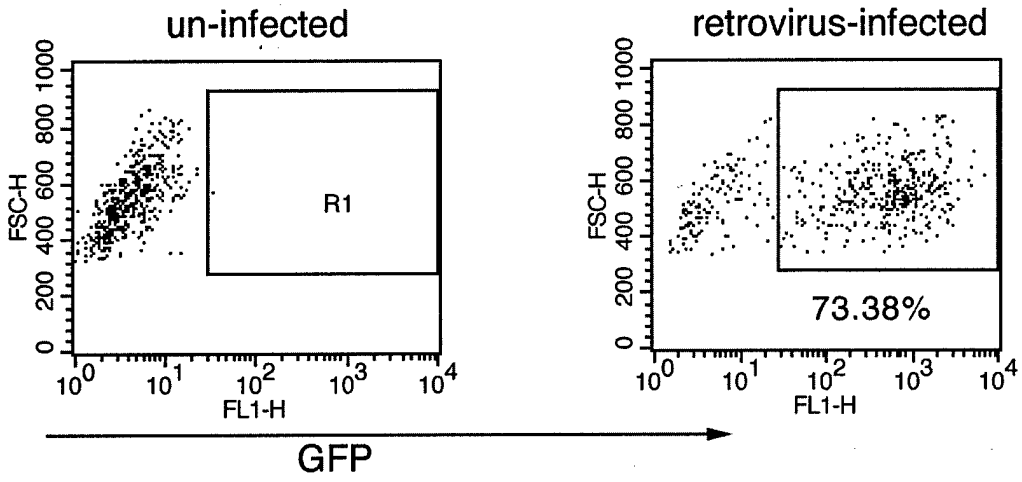


Fig. 3 Bone marrow-derived dendritic cells were infected with MIG retrovirus supernatant on day 2 and day 4. GFP expression was analyzed 48 hours after infection.

Having generated the MIG-IKK virus, we were also interested in determining its effect on mouse splenic CD4 T cells. Similar to DCs, we have found that NF-kB proteins also play an important role in regulating survival of CD4 T cells. Likely because of the high rate of proliferation in activated T cells, these cells could be easily infected by retroviruses without requiring much optimization. To provide additional support for a role of NF-kB in regulating T cell survival, we infected them with the MIG-IKK retrovirus. Significantly, MIG-IKK infection was sufficient to enhance survival of CD4 T cells. In contrast, survival of T cells lacking the p50 and cRel subunits of NF-kB, thus having very little NF-kB activity, could not be enhanced by MIG-IKK. These results demonstrate that MIG-IKK requires NF-kB activity for enhancement of survival, as we would expect. These results were recently published in the *Journal of Experimental Medicine* (4). Although they are not directly related to the proposed studies, these results demonstrate the efficacy of MIG-IKK in enhancing survival of a primary cell type, a key aspect of the proposed study.

We want to ensure that upon completion, these studies would have helped determine whether constitutive activation of NF-kB in DCs can be used as a method for enhancing T cell responses, including those against tumor cells. We have therefore also initiated identical studies using a lentiviral transduction approach. We believe this approach is as good, and in some respects, better than the retroviral approach we are currently using. Thus, lentiviruses can also infect not dividing cells, and can be concentrated by ultracentrifugation. This should allow us to perform a single infection at the end of the DC generation period with high MOI lentiviral supernatants. As shown in Fig. 4, a single lentiviral infection on day 4 led to infection of approximately 50% of DCs. We are now also sub-cloning CA-IKKβ into lentiviral vectors. In our continuing studies, we will determine the efficacy of both retroviral and lentiviral expression of CA-IKKβ in DCs on their survival and T cell activation functions.

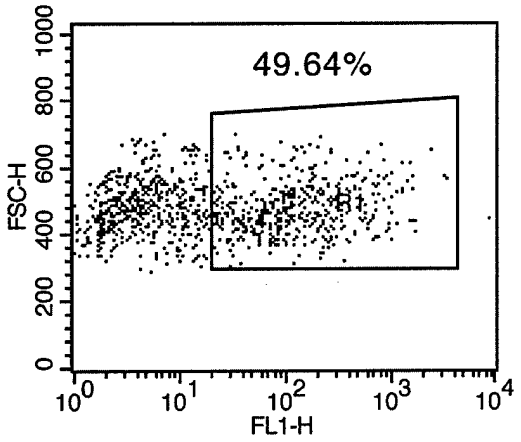


Fig. 4 Bone marrow-derived dendritic cells were infected with lentivirus supernatants on day 4 (M.O.I. (multiplication of infection) =5). GFP expression was analyzed 48 hours after infection.

Key research accomplishments:

- Our studies demonstrate the feasibility of infecting DCs with retroviral and lentiviral vectors
- Our findings indicate that CA-IKKβ expression is sufficient to activate NF-kB in mouse fibroblasts. Similar studies are now being conducted on DCs

Reportable Outcomes

Manuscripts: Retroviral constructs generated as part of this study were used for studies on primary mouse T cells. These studies led to a manuscript, which was published in the *The J.Exp. Med.* recently (enclosed in appendix). Also enclosed in the appendix is our manuscript on NF-kB function in DCs.

Conclusions

Our studies have demonstrated the feasibility of infecting DCs with retroviral and lentiviral vectors. Our findings also indicate that CA-IKKβ expression is sufficient to activate NF-kB in mouse fibroblasts. Similar studies are now being performed on DCs. In T cells, we have shown that CA-IKKβ expression is sufficient to promote survival, in an NF-kB dependent manner. Based on these findings, we are poised to carry out studies aimed at determining how expression of CA-IKKβ in DCs impacts their survival and T cell responses in vitro and in vivo. These studies will help determine the feasibility of our approach for potentiating T cell responses against tumor cells.

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Appendices:

1) Ouaaz, F., J. Arron, Y. Zheng, Y. Choi, and A.A. Beg. 2002. Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity* 16:257-270.

2) Zheng, Y., M. Vig, J. Lyons, L. Van Parijs, and A.A. Beg. 2003. Combined deficiency of p50 and cRel in CD4+ T cells reveals an essential requirement for NF-kB in regulating mature T cell survival and in vivo function. *J Exp Med* 197:861-874.

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Dendritic Cell Development and Survival Require Distinct NF-κB Subunits

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Summary

Despite the established role of dendritic cells (DCs) in regulating T lymphocyte activation, intracellular mechanisms responsible for controlling DC function are largely undefined. Here, we have studied DCs from mice deficient in the p50, RelA, and cRel subunits of the immunomodulatory NF-κB transcription factor. Although DC development and function was normal in mice lacking individual NF-κB subunits, development of doubly deficient $p50^{-/-}RelA^{-/-}$ DCs was significantly impaired. In contrast, DCs from $p50^{-/-}cRel^{-/-}$ mice developed normally, but CD40L- and TRANCE-induced survival and IL-12 production was abolished. Surprisingly, no significant impairment in MHC and costimulatory molecule expression was seen, despite significantly reduced κB site binding activity. These results therefore indicate essential, subunit-specific functions for NF-κB proteins in regulating DC development, survival, and cytokine production.

Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that play an essential role in activation of T lymphocytes (Banchereau et al., 2000; Banchereau and Steinman, 1998). Among APCs, which also include macrophages and B cells, only DCs are believed to be capable of activating naive T cells. DC function is regulated by their state of maturation. Originating from both myeloid and lymphoid hematopoietic precursors in the bone marrow, DCs migrate to the periphery as "immature" cells (Banchereau et al., 2000; Banchereau and Steinman, 1998; Wu et al., 2001). "Maturation" of DC can be induced by microbial stimuli, proinflammatory cytokines, as well as through interaction with CD40L-expressing T cells (Banchereau et al., 2000; Banchereau and Steinman, 1998; Caux et al., 1994; Reis e Sousa, 2001). Mature DCs are highly immunogenic due to high levels of expression of MHC I, II, costimulatory, and adhesion molecules, including B7-1, B7-2, CD40, and ICAM-1. Interaction of DCs with microbial agents, such as lipopolysaccharide (LPS) or CD40L, also induces IL-12 production, a cytokine that regulates Th cell differentiation into Th1 cells (Trinchieri, 1998). Mature DCs preferentially migrate to the T cell areas of secondary lymphoid tissues where they can induce activation and proliferation of naive T-helper and T-cytotoxic lymphocytes (CTLs) (Banchereau et al., 2000). Several distinct DC subsets originating from both myeloid and lymphoid precursors have now been identified (Shortman, 2000; Traver et al., 2000; Wu et al., 2001). Of these, $CD11c^+CD8\alpha^-$ and $CD11c^+CD8\alpha^+$ comprise the two major subsets whose specific functions are being extensively studied.

The control of DC survival plays an important role in regulating their T cell priming functions (Josien et al., 2000; Wong et al., 1997). However, DCs in mice have been shown to have a very short life span (Kamath et al., 2000). Significantly, engagement of CD40 expressed on DCs with CD40L expressed on T cells not only stimulates maturation and cytokine production but also enhances DC survival (Caux et al., 1994; Miga et al., 2001). The effect of enhanced DC survival in potentiating T cell activation has been best demonstrated by studies of TRANCE, a TNF family member expressed on T cells that promotes DC survival but not maturation (Josien et al., 2000; Wong et al., 1997). These studies have established a role for CD40L and TRANCE in regulation of DC survival, although the intracellular survival pathways involved have yet to be defined.

Although the significance of DCs as regulators of adaptive immunity is beyond doubt, little is known about intracellular mechanisms specifically responsible for regulating DC function and survival. Interestingly, many inducers of DC maturation are also strong activators of NF-κB transcription factors (Baldwin, 1996; Ghosh et al., 1998), suggesting that these factors may play a key role in DC maturation. The NF-κB family of transcription factors exist as homodimers or heterodimers of five distinct proteins (p50, p52, RelA, RelB, and cRel) and play an important role in regulating inflammatory and immune-response genes (Ghosh et al., 1998). NF-κB activation occurs by nuclear translocation following inducible phosphorylation of inhibitory IκB proteins by the IKKβ (IκB kinase) complex (Karin and Ben-Neriah, 2000).

All five members of the NF-κB family have been knocked out in mice (Beg et al., 1995; Burkly et al., 1995; Caamano et al., 1998; Doi et al., 1997; Franzoso et al., 1998; Kontgen et al., 1995; Sha et al., 1995; Weih et al., 1995). These studies have identified key roles for NF-κB proteins in regulation of innate immunity, lymphocyte function, and regulation of cell survival (Alcamo et al., 2001; Beg et al., 1995; Grumont et al., 1999; Kontgen et al., 1995; Ouaaz et al., 1999; Sha et al., 1995; Zheng et al., 2001). Studies of $RelB^{-/-}$ mice have indicated a specific requirement for this protein in development of $CD11c^+CD8\alpha^-$ but not $CD11c^+CD8\alpha^+$ DCs (Burkly et al., 1995; Weih et al., 1995; Wu et al., 1998). However, other than RelB, the function of other NF-κB subunits in DCs is not known.

We show here that κB site binding complexes in DCs consist in large part of p50, RelA, and cRel subunits. To understand the function of these proteins in DCs, we have utilized mice deficient in RelA, p50, and cRel (Beg

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et al., 1995; Kontgen et al., 1995; Sha et al., 1995). We demonstrate here that the absence of these individual subunits does not affect DC survival, maturation, or T cell stimulatory function. However, profound defects in DC development and mature DC function were found in the combined absence of p50+RelA or p50+cRel, respectively. Based on the results presented here, we propose a key function of NF-κB complexes comprising of p50, RelA, and cRel in regulation of DC development, survival, and IL-12 production.

Results

Composition of κB Site Binding Complexes in Dendritic Cells

To study NF-κB function in dendritic cells (DCs), we first determined subunit composition of κB site binding complexes present in bone marrow (BM)-derived DCs. EMSA analysis was performed with the high-affinity H-2 κB site, in order to detect the maximal number of DNA binding-competent NF-κB complexes in DCs. As shown in Figure 1A, constitutive κB site binding activity was strongly increased when DCs were treated with microbial LPS, one of the best known DC maturation-inducing agents. LPS treatment induced two distinct κB site binding complexes. Antisera generated against p50 and cRel significantly inhibited these complexes, while antisera generated against RelA had a less significant effect. These results thus indicate that NF-κB complexes in dendritic cells consist of significant levels of p50 and cRel but do not preclude presence or involvement of additional NF-κB subunits (e.g., p52 and RelB) in LPS-induced DC responses.

Lack of RelA, p50, or cRel Does Not Affect Development or Maturation of Dendritic Cells

To determine the possible function of RelA, p50, and cRel NF-κB subunits in development and function of DCs, we analyzed DCs from mice specifically lacking each of these proteins. Since RelA$^{-/-}$ mice die at embryonic day 15 (Beg et al., 1995), we first generated chimeric mice following adoptive-transfer of control or RelA$^{-/-}$ fetal liver (FL) hematopoietic cells (CD45.2-expressing) into lethally irradiated CD45.1-expressing mice. To determine a role for RelA in DC development, spleen DCs were obtained from RelA$^{-/-}$ FL-transplanted mice. Two months after transplantation, cells displaying typical stellate DC morphology were obtained from spleens of both control and RelA$^{-/-}$ FL-transplanted mice. These cells were CD45.2-positive, demonstrating their origin from donor FL hematopoietic cells, and also expressed the DC-specific integrin CD11c (Figure 1B). Previous studies have shown that the NF-κB family member RelB is required for development of CD11c$^+$CD8α$^-$ but not CD11c$^+$CD8α$^+$ DCs (Wu et al., 1998). In contrast, both CD11c$^+$CD8α$^-$ and CD11c$^+$CD8α$^+$ DCs were generated in the absence of RelA (Figure 1B). These results demonstrate that unlike RelB, RelA is not required for development of specific DC subsets.

Consistent with relatively low reactivity of RelA antisera, total κB site binding activity was not significantly reduced in RelA$^{-/-}$ DCs (data not shown). Since EMSA analyses showed significant levels of both p50 and cRel, we next analyzed DC development in p50$^{-/-}$ and cRel$^{-/-}$ mice. Similar to RelA$^{-/-}$ mice, spleen DCs from both p50$^{-/-}$ and cRel$^{-/-}$ also showed the presence of CD11c$^+$CD8α$^-$ and CD11c$^+$CD8α$^+$ DCs (Figure 1C). BM-derived DCs have high expression of MHC and costimulatory molecules and can prime T lymphocytes in vitro and in vivo (Inaba et al., 1990; Sigal et al., 1999). To determine whether RelA, p50, or cRel subunits are required for expression of T cell stimulatory molecules in DCs, we derived DC from BM cells of these mice. Adherent clusters of immature DCs, and cells with characteristic DC morphology were obtained from wild-type, RelA$^{-/-}$, p50$^{-/-}$, and cRel$^{-/-}$ mice, which expressed CD11c (data not shown). Importantly, DCs obtained from different subunit-deficient mice showed normal constitutive and LPS-induced expression of MHC I (H-2K) and II (I-A), B7-2, and ICAM-1 (data not shown). These results thus indicate that RelA, p50, or cRel subunits are not absolutely essential for expression of MHC and costimulatory molecules in DCs.

Allogeneic stimulation of T cells in a mixed leukocyte reaction (MLR) is one of the defining properties of DCs (Inaba et al., 1992). We next determined a possible role for RelA, p50, and cRel in allogeneic T cell stimulation. To this end, subunit-deficient BM DCs ("*b*" MHC haplotype) were γ irradiated and incubated with T cells from BALB/c mice ("*d*" MHC haplotype). In all cases, unstimulated and LPS-stimulated DCs induced robust T cell proliferative responses (Figure 1D). These results thus demonstrate that the individual absence of RelA, p50, or cRel does not affect DC development, maturation, or T cell stimulation.

Impaired Dendritic Cell Development in the Combined Absence of p50 and RelA

The lack of phenotypic defects in RelA, p50, or cRel DCs may be because of redundancy in function of these NF-κB subunits. This possibility was tested by studying DC development and function in mice lacking more than one NF-κB subunit. Of particular interest was to determine DC function in mice that are completely deficient in the two major NF-κB heterodimers, p50+RelA and p50+cRel. The consequence of absence of p50+RelA was investigated first. Chimeric mice were generated by adoptive-transfer of fetal liver cells into lethally irradiated CD45.1-expressing mice, as described above for RelA$^{-/-}$ mice. Four to five weeks after transfer, significant numbers of CD11c$^+$ DCs were obtained from p50$^{-/-}$RelA$^{+/-}$ FL-transplanted mice. Strikingly, virtually no CD11c$^+$ cells were detected in spleens of mice transplanted with p50$^{-/-}$RelA$^{-/-}$ (Figure 2A). To determine whether the absence of CD11c$^+$ cells was indicative of the absence of DCs or impaired CD11c expression, spleen cytospin preparations from control and p50$^{-/-}$RelA$^{-/-}$ mice were analyzed. No cells with characteristic DC morphology were obtained from p50$^{-/-}$RelA$^{-/-}$-transplanted mice, while control mice showed many typical DCs (Figure 2B). These results demonstrate that redundant functions of p50 and RelA include a potentially critical role in DC generation. Significantly, the virtual absence of DCs indicates a role for p50+RelA in generation of both CD11c$^+$CD8α$^-$ and CD11c$^+$CD8α$^+$ DCs. Monocytes/macrophages and DCs originating from myeloid precursors share common he-

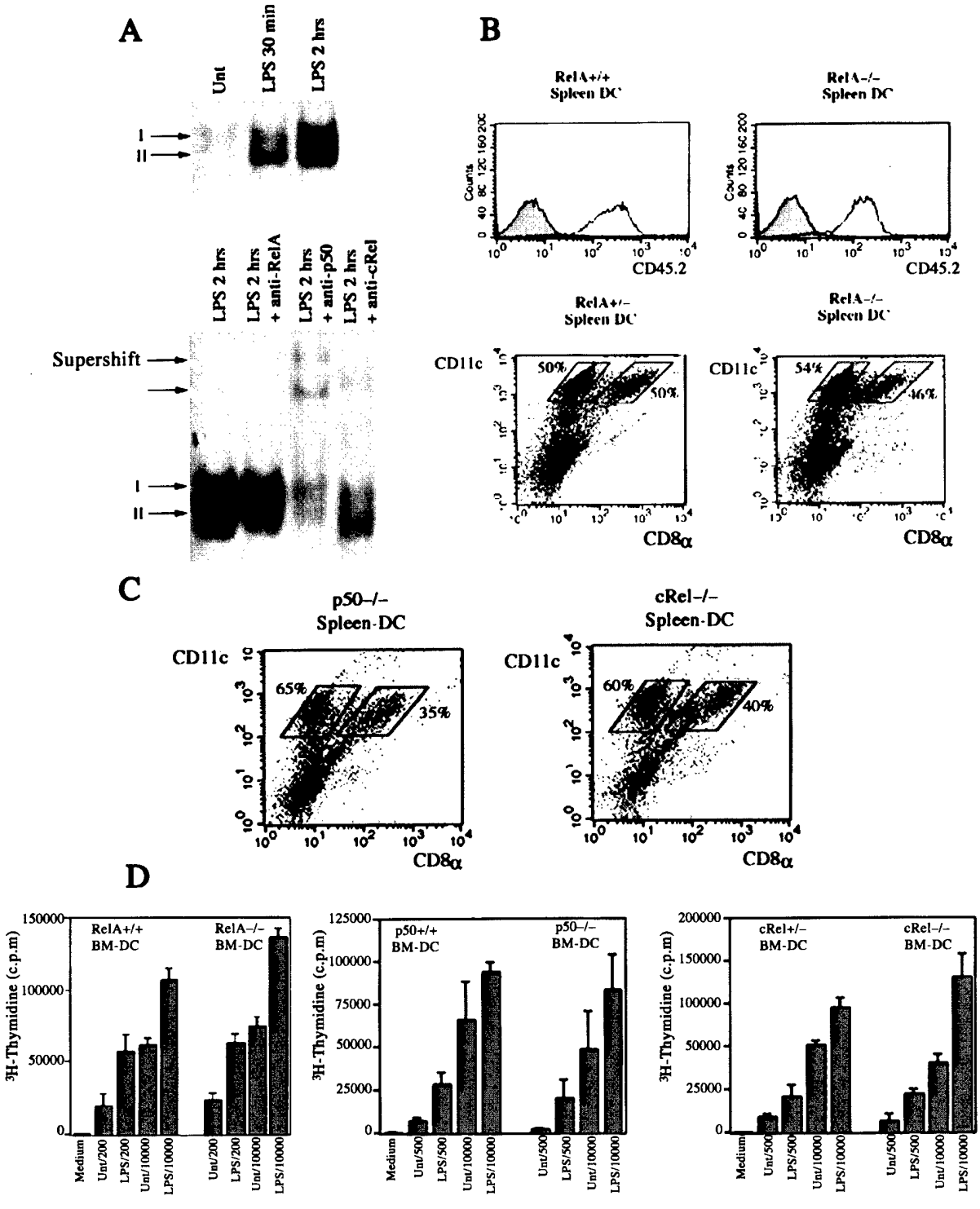


Figure 1. Normal DC Development in RelA$^{-/-}$, p50$^{-/-}$, and cRel$^{-/-}$ Mice

(A) NF-κB/Rel subunit composition in dendritic cells. Bone marrow-derived dendritic cells were left untreated or stimulated for 30 min and 2 hr with LPS, nuclear extracts prepared and analyzed by EMSA for binding to the H-2K κB site (top panel). Nuclear extracts were also preincubated with anti-RelA (p65), anti-p50, and anti-cRel antibodies prior to incubation with the κB site oligonucleotide probe (bottom panel)

(B) Immunostaining of spleen-derived dendritic cells from RelA$^{-/-}$ fetal liver-transplanted mice. Spleen DCs were isolated from mice transplanted with control or RelA$^{-/-}$ fetal liver cells. DCs were left unstained (shaded histogram) or stained with FITC-conjugated antibodies against murine CD45.2 and CD8α and with PE-conjugated antibody against CD11c for analysis by FACS. CD11c^{+}CD8α^{-} cells are shown in the left rectangle while CD11c^{+}CD8α^{+} are shown in the right rectangle, along with respective percentages.

(C) Immunostaining of spleen-derived DCs from cRel- and p50-deficient mice. Spleen DCs were isolated from wild-type, cRel-, and p50-deficient mice. DCs were left unstained or stained with FITC-conjugated antibody against murine CD8α and with PE-conjugated antibody against CD11c for analysis by FACS. CD11c^{+}CD8α^{-} cells are shown in the left rectangle while CD11c^{+}CD8α^{+} are shown in the right rectangle, along with respective percentages.

(D) Allogeneic MLR-mediated by RelA$^{-/-}$, cRel$^{-/-}$, and p50$^{-/-}$ bone marrow-derived DCs. CD3^{+} T cells (100,000) from BALB/c mice were incubated with γ-irradiated bone marrow dendritic cells (numbers indicated). DCs were left untreated or treated with LPS for 16 hr as indicated. After 3 days of culture, ^{3}H-thymidine incorporation was determined.

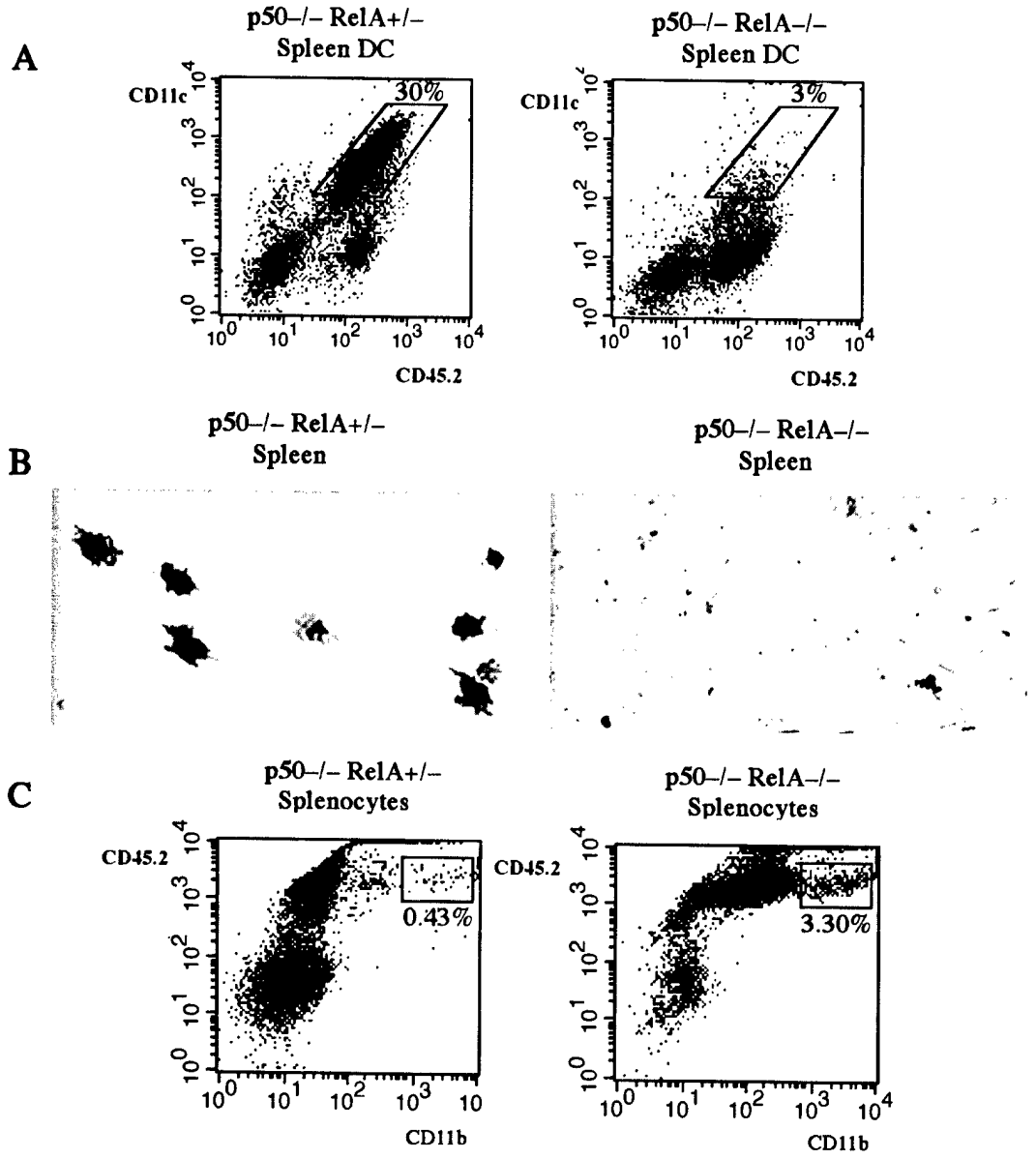


Figure 2. Impaired Spleen DC Development in p50$^{-/-}$RelA$^{-/-}$ FL-Transplanted Mice

(A) Immunostaining of spleen DCs from p50$^{-/-}$RelA$^{+/-}$ and p50$^{-/-}$RelA$^{-/-}$ mice. Spleen DCs were isolated from mice transplanted with p50$^{-/-}$RelA$^{+/-}$ or p50$^{-/-}$RelA$^{-/-}$ fetal liver cells. DCs were left unstained or stained with FITC-conjugated antibody against murine CD45.2 and with PE-conjugated antibody against CD11c for analysis by FACS. CD11c^{+}CD45.2^{+} cells are shown along with respective percentages.

(B) Cytospin-preparation analysis from p50$^{-/-}$RelA$^{+/-}$ and p50$^{-/-}$RelA$^{-/-}$ spleens. DCs were isolated from spleens as described above. After overnight culture, nonadherent cells were collected. Heme 3-stained cytospin preparations are shown.

(C) Immunostaining of p50$^{-/-}$RelA$^{+/-}$ and p50$^{-/-}$RelA$^{-/-}$ splenocytes. Low-density splenocytes were prepared from whole spleens. Cells were left unstained or were stained with FITC-conjugated antibody against murine CD11b (Mac1) and with PE-conjugated antibody against CD45.2 for analysis by FACS. CD11b high macrophages are shown in the rectangles. Because p50$^{-/-}$RelA$^{-/-}$ splenocytes have a significantly higher proportion of both CD11b high and CD11b low cells (monocytes), only 25% of total events are shown to clearly distinguish between these populations. The CD11b population shown indicates cells released by mechanical disruption only, rather than by more efficient enzymatic treatment.

matopoietic precursors (Wu et al., 2001). We therefore determined whether p50+RelA were also required for macrophage generation. As shown in Figure 2C, CD11b^{+} high (Mac1^{+}) monocyte/macrophages were readily detected in splenocyte preparations of p50$^{-/-}$RelA$^{-/-}$ FL-transplanted mice. The significantly higher relative percentage of p50$^{-/-}$RelA$^{-/-}$ splenic macrophages (only 25% of total events are shown for p50$^{-/-}$RelA$^{-/-}$ mice) is likely due to impaired lymphocyte generation (see below) rather than an absolute increase in numbers of macrophages. Macrophages were also detected in cytospin preparations and by FACS analysis of peritoneal cells from p50$^{-/-}$RelA$^{-/-}$ FL-transplanted mice (data not shown). These results therefore indicate a specific re-

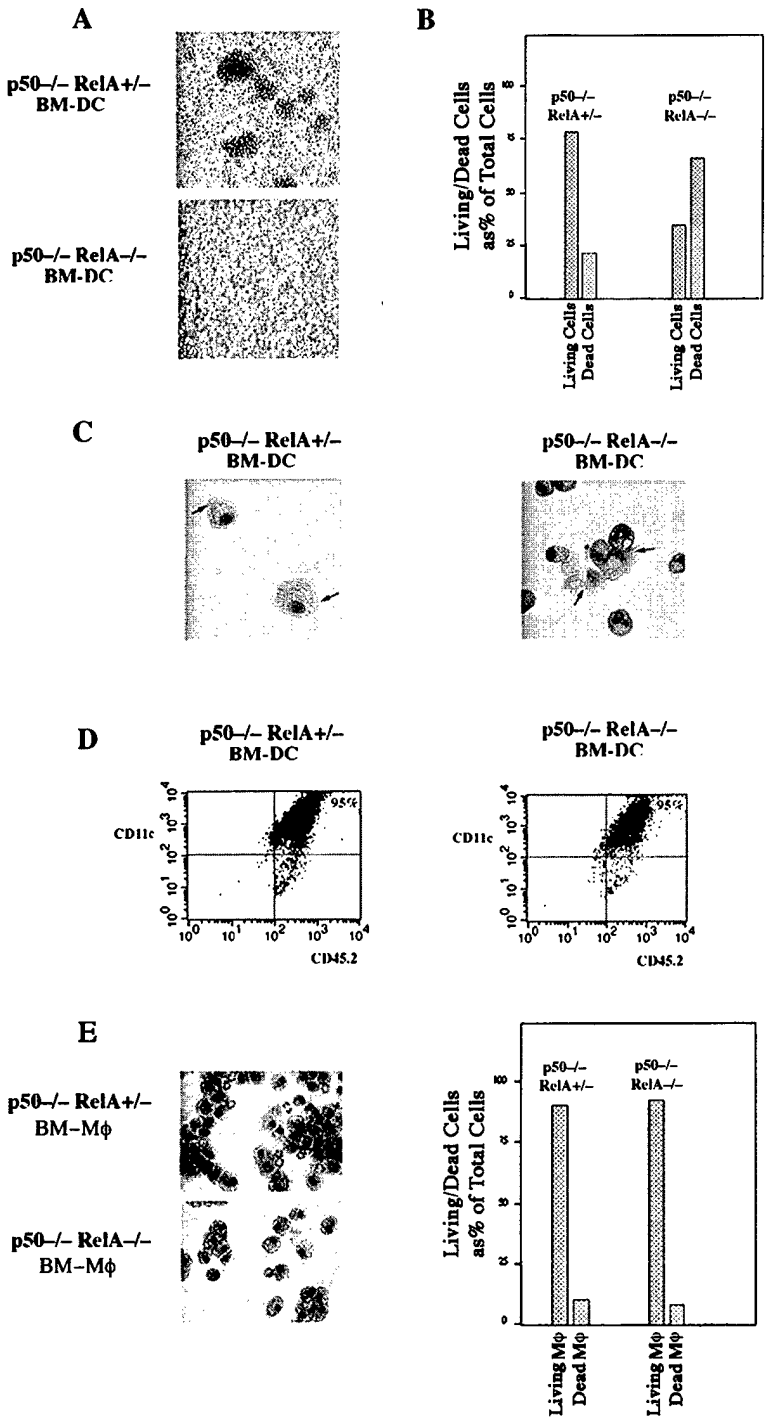


Figure 3. Analysis of $p50^{-/-}RelA^{-/-}$ Bone Marrow-Derived DCs

(A) Generation of bone marrow-derived DCs from fetal liver $p50^{-/-}RelA^{+/-}$- and $p50^{-/-}RelA^{-/-}$-transplanted mice. Bone marrow (BM) cells were used for generation of DCs. After 3–4 days of culture, clusters of developing DCs were analyzed by light microscopy.

(B) Cell death analysis of developing bone marrow-derived $p50^{-/-}RelA^{+/-}$ and $p50^{-/-}RelA^{-/-}$ DCs. On day 4 of culture, cells are harvested and cell death quantified by measuring cell viability by Trypan blue exclusion. Dead and living cells are shown as a percentage of total cells.

(C) Cytospin analysis of bone marrow-derived $p50^{-/-}RelA^{+/-}$ and $p50^{-/-}RelA^{-/-}$ DCs. On day 6 of culture, DCs were harvested and cytospin preparations were analyzed.

(D) Immunostaining of bone marrow-derived $p50^{-/-}RelA^{+/-}$ and $p50^{-/-}RelA^{-/-}$ DCs. On day 6 of culture, DCs were harvested, washed, and stained with FITC-conjugated antibody against CD45.2 and with PE-conjugated antibody against CD11c for FACS analysis.

(E) Analysis of morphology and cell death of bone marrow-derived macrophages from FL $p50^{-/-}RelA^{+/-}$- and $p50^{-/-}RelA^{-/-}$-transplanted mice. Bone marrow-derived macrophages were generated from bone marrow cells in the presence of recombinant M-CSF. Cytospin preparations are shown (left panel). Bone marrow-derived macrophages were harvested and cell viability determined by Trypan blue exclusion. Dead and living cells are shown as a percentage of total cells (right panel).

quirement for p50+RelA in generation of DCs but not macrophages.

To further understand the mechanism responsible for impaired DC generation, BM cells were used for generation of DCs from $p50^{-/-}RelA^{+/-}$- and $p50^{-/-}RelA^{-/-}$-transplanted mice. Within 3 days, clusters of immature DCs could be detected in cultures of $p50^{-/-}RelA^{+/-}$ BM cells. However, virtually no such clusters were detected when $p50^{-/-}RelA^{-/-}$ BM cells were cultured under the same conditions (Figure 3A). Significantly, $p50^{-/-}RelA^{-/-}$ BM cultures revealed large numbers of dead cells, although some cells with characteristic DC morphology were also present. On day 4, approximately 70% of the cells were dead in $p50^{-/-}RelA^{-/-}$, while only 20% dead cells were detected in $p50^{-/-}RelA^{+/-}$ cultures (Figure 3B). As a result of this massive cell death, the yield of $p50^{-/-}RelA^{-/-}$ DCs was typically 10% that of control DCs. However, the small numbers of surviving cells had typical DC morphology (Figure 3C) and CD11c expression (Figure 3D).

DCs obtained from BM GM-CSF cultures are of myeloid origin and share common myeloid precursors with monocyte/macrophages (Inaba et al., 1993). The low numbers of $p50^{-/-}RelA^{-/-}$ $CD11c^+$ cells obtained were also $CD11b^+$, indicating their myeloid origin (data not shown). We then determined whether impaired in vitro DC generation could potentially be due to reduced survival of $p50^{-/-}RelA^{-/-}$ common myeloid precursors. To this end, we tested the ability of $p50^{-/-}RelA^{-/-}$ myeloid precursors to differentiate into macrophages. In striking contrast to DC cultures, $p50^{-/-}RelA^{-/-}$ BM cells readily differentiated into macrophages in the presence of M-CSF (Figure 3E). Significantly, no enhanced susceptibility to cell death was noticed in these cultures (Figure 3E), and similar numbers of macrophages were obtained from control and $p50^{-/-}RelA^{-/-}$ BM cells. These results indicate that impaired DC generation is not due to reduced numbers and/or survival of myeloid precursors in BM cultures. Together with in vivo studies, these results provide additional evidence for an essential role for p50+RelA in DC development. These results also indicate that impaired DC development may be due to high susceptibility to cell death in the absence of p50+RelA.

Rescue of Lymphocyte but Not DC Generation in $p50^{-/-}RelA^{-/-}$ Mice by Wild-Type Hematopoietic Precursors

Previous studies have shown impaired lymphocyte generation in mice following adoptive transfer of $p50^{-/-}RelA^{-/-}$ FL cells (Horwitz et al., 1997). However, normal generation of p50+RelA-deficient B cells was shown to occur following culture of hematopoietic precursors in vitro (Horwitz et al., 1997). Significantly, failure of lymphopoiesis could be rescued by cotransplantation of mice with wild-type (CD45.1) bone marrow cells, indicating that this defect was not cell autonomous. To determine whether $p50^{-/-}RelA^{-/-}$ DC generation could be similarly rescued, $p50^{-/-}RelA^{-/-}$ FL cells (CD45.2) were cotransplanted with wild-type CD45.1 bone marrow cells. As shown in Figure 4A, defective $p50^{-/-}RelA^{-/-}$ B lymphocyte generation was rescued following coinjection of CD45.1 cells. These results indicate normal generation of common lymphoid precursors potentially capable of differentiating into lymphocytes or DCs in these mice. However, generation of $p50^{-/-}RelA^{-/-}$ $CD11c^+$ DCs (CD45.2) remained impaired, while CD45.1 CD11c DCs were readily generated (Figure 4B). These results further underscore a requirement for p50+RelA in generation of DCs and provide evidence for a cell-autonomous function for these proteins in DC generation.

Significant Reduction in κB Site Binding Activity in $p50^{-/-}cRel^{-/-}$ DCs Does Not Affect Development or Maturation but Abolishes IL-12 Expression

In addition to p50+RelA, p50+cRel heterodimers constitute the other main NF-κB complex in several cell types, and as shown in Figure 1A, may comprise the major NF-κB complex in DCs. In order to determine the effect of absence of p50 and cRel subunits, we first analyzed spleen DC development in $p50^{-/-}cRel^{-/-}$ mice. Significantly, unlike $p50^{-/-}RelA^{-/-}$ mice, $p50^{-/-}cRel^{-/-}$ mice showed normal DC generation and the presence of both $CD11c^+CD8\alpha^-$ and $CD11c^+CD8\alpha^+$ DCs (Figure 5A). Thus, DC development appears specifically dependent on p50+RelA but not p50+cRel.

As shown in Figure 1A, p50- and cRel-containing complexes constitute a major proportion of the total κB site binding activity in LPS-treated DCs. As shown in Figure 5B, compared to BM-derived DCs lacking p50 or cRel subunits, both untreated and LPS-treated DCs from $p50^{-/-}cRel^{-/-}$ mice showed dramatically reduced κB site binding activity. To determine whether normal DC maturation in the absence of p50 or cRel may be due to redundancy in function of these proteins, we investigated whether DC maturation induced by LPS was affected in $p50^{-/-}cRel^{-/-}$ DCs. Interestingly, other than a small decrease in expression of MHC class II I-A, no significant decrease in basal expression of MHC I, B7-1, B7-2, and ICAM-1 was noticed in $p50^{-/-}cRel^{-/-}$ DCs. Upon LPS treatment, expression of both MHC I/II and costimulatory molecules was strongly and similarly enhanced in wild-type and $p50^{-/-}cRel^{-/-}$ DCs (Figure 5C). Furthermore, identical results were obtained following CD40L treatment of $p50^{-/-}cRel^{-/-}$ DCs (Figure 5C). Thus, significantly reduced κB site binding activity in these DCs does not appear to impair expression of MHC and costimulatory molecules. Despite apparently normal maturation, allogeneic T cell proliferation induced by $p50^{-/-}cRel^{-/-}$ DCs was reduced compared to wild-type DCs (Figure 5D). On closer examination, we found this was due to significantly reduced survival of $p50^{-/-}cRel^{-/-}$ DCs following γ irradiation (data not shown). However, γ irradiation-induced cell death was significantly inhibited following LPS treatment (by an undefined but likely NF-κB-independent mechanism), which also restored $p50^{-/-}cRel^{-/-}$ DC MLR induction to levels similar to wild-type DCs. Taken together, these results indicate apparently normal maturation and T cell stimulation in the combined absence of p50+cRel subunits.

Previous studies have indicated a potentially important role for NF-κB proteins in regulating IL-12 expression (Yoshimoto et al., 1997). Interaction of DCs with LPS and CD40L results in potent induction of biosynthesis of IL-12 p70, which is generated by dimerization of two independent subunits called p40 and p35 (Trinchieri, 1998). Transcription of both p40 and p35 is induced by LPS and CD40L, likely through interaction of multiple transcription factors, including PU.1, IRF-1, and NF-κB, with regulatory control elements (Trinchieri, 1998). To determine whether NF-κB proteins are essential for IL-12 expression, we utilized DCs from wild-type, $p50^{-/-}$, $RelA^{-/-}$, $cRel^{-/-}$, and $p50^{-/-}cRel^{-/-}$ mice. Northern analysis of p40 mRNA expression, however, showed no significant reduction in expression in $p50^{-/-}$, $RelA^{-/-}$, and $cRel^{-/-}$ DCs treated for 6 hr with LPS, in comparison with control DCs (Figure 6A). In contrast, LPS-induced p40 expression was dramatically reduced in $p50^{-/-}cRel^{-/-}$ DCs (Figure 6A). Furthermore, CD40L induction of p40 was also reduced in $p50^{-/-}cRel^{-/-}$ DCs. We then tested induction of IL-12 p70 by ELISA using culture supernatants of control and $p50^{-/-}cRel^{-/-}$ DCs treated with LPS. While IL-12 production was significantly induced in control cells, no detectable IL-12 was produced following LPS treatment of $p50^{-/-}cRel^{-/-}$ DCs (Figure 6B).

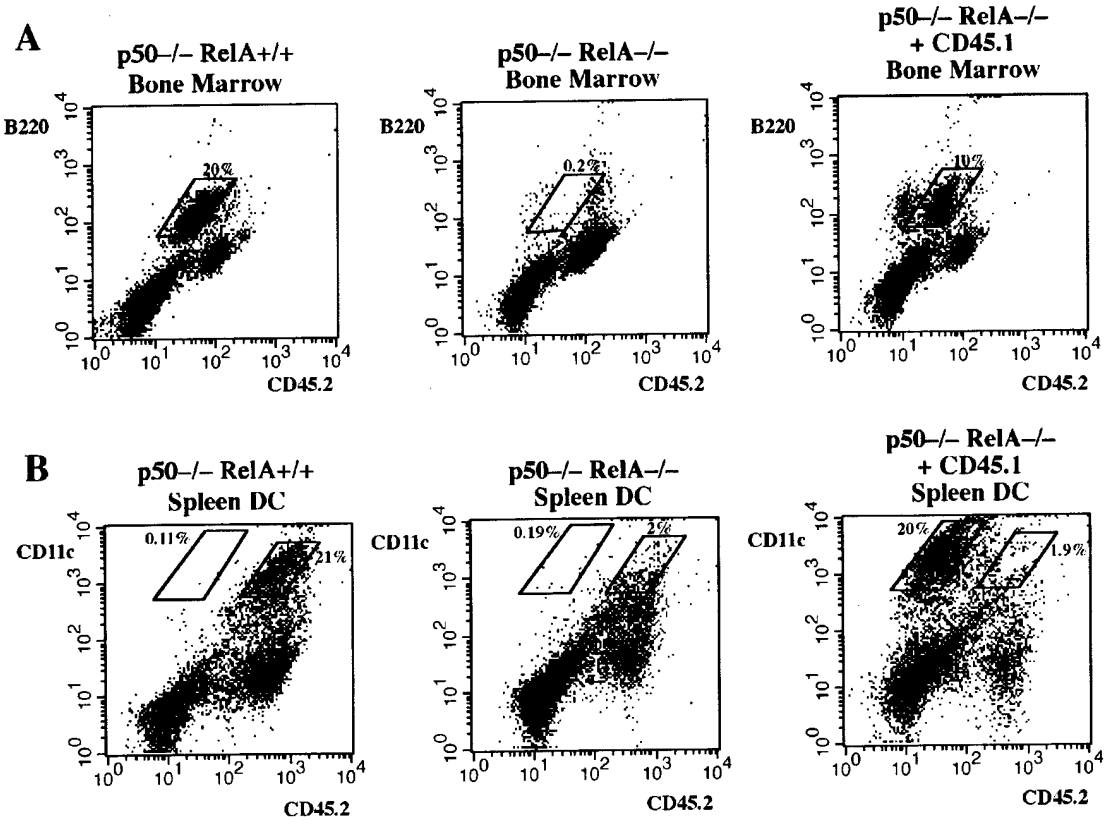


Figure 4. Analysis of Spleen DC and B Lymphocyte Development in CD45.1-Coinjected Mice

(A) Immunostaining of bone marrow cells from FL $p50^{-/-}RelA^{+/+}$-, $p50^{-/-}RelA^{-/-}$-, and CD45.1-coinjected mice. Bone marrow cells were collected and stained with PE-conjugated antibody against B220 and with FITC-conjugated antibody against CD45.2 for FACS analysis.

(B) Immunostaining of spleen-collagenase preparations from FL $p50^{-/-}RelA^{+/+}$-, $p50^{-/-}RelA^{-/-}$-, and CD45.1-coinjected mice. Cells were stained with PE-conjugated antibody against CD11c and with FITC-conjugated antibody against CD45.2 for FACS analysis. $CD45.2^{+}$ cells, along with percentages, are shown in the right rectangle.

Similarly, CD40L-induced IL-12 production was also abolished in $p50^{-/-}cRel^{-/-}$ DCs (Figure 6B). These results thus demonstrate an essential role for p50+cRel in LPS- and CD40L-induced expression of IL-12.

Regulation of Mature DC Survival by CD40L, TRANCE, and NF-κB p50+cRel

DC survival can be enhanced following engagement of CD40 or TRANCE-R. Previous studies have shown that CD40 and TRANCE-R activate multiple signaling pathways, including PI3K/Akt and NF-κB (Arron et al., 2001; Wong et al., 1999). Based on results showing a potentially important role for p50+RelA in developing DC survival, we determined whether mature DC survival was also regulated by NF-κB proteins. To this end, we tested the effect of CD40L treatment on survival of different NF-κB-deficient DCs. Mature BM-derived DCs obtained from wild-type, $p50^{-/-}$, $RelA^{-/-}$, $cRel^{-/-}$, and $p50^{-/-}cRel^{-/-}$ mice were incubated in the absence or presence of CD40L for 5 days. DC survival was substantially enhanced in wild-type DCs (approximately 6-fold) and in DCs lacking RelA, p50, and cRel (Figure 7A). Significantly, $p50^{-/-}cRel^{-/-}$ DCs were almost completely impaired in CD40L-induced enhancement of survival (Figure 7A), although both control and $p50^{-/-}cRel^{-/-}$ DCs were found to have similar expression of CD40 (Figure 7B). In addition, we have also found that survival of TRANCE-treated $p50^{-/-}cRel^{-/-}$ DCs was also significantly lower than that of control DCs (Figure 7A). CD40L treatment of BM-derived DCs induced high NF-κB levels for up to 36 hr, which were significantly reduced in $p50^{-/-}cRel^{-/-}$ BM DCs (Figure 7C). Thus, similar to LPS, CD40L-induced κB activity also consists in large part of p50+cRel subunits. Induction of antiapoptotic Bcl-2 family member expression, in particular Bcl-xL, may be responsible for inhibition of cell death by CD40L and TRANCE (Wong et al., 1997). Furthermore, ectopic Bcl-xL expression may be sufficient for enhancement of DC survival (Pirtskhalaishvili et al., 2000). As shown in Figure 7D, Bcl-xL expression was strongly induced in wild-type but not in CD40L-treated $p50^{-/-}cRel^{-/-}$ DCs. We conclude that activation of p50+cRel complexes plays an essential role in regulating survival of mature DCs.

Discussion

Studies carried out over the past several years have demonstrated the key role played by DCs in regulating T cell activation. However, signaling pathways involved in regulating specific aspects of DC function are still poorly understood. To this end, we have carried out a

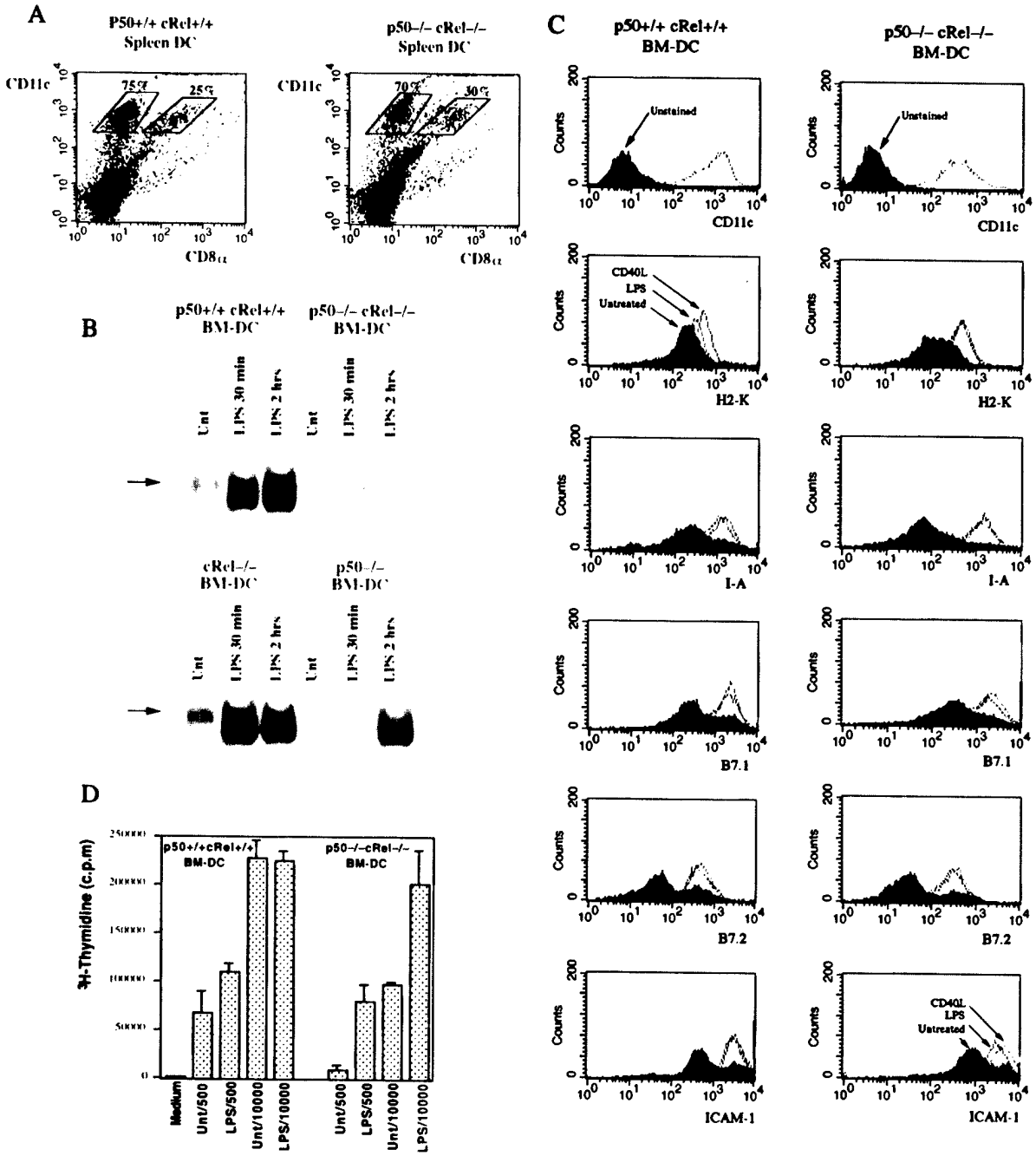


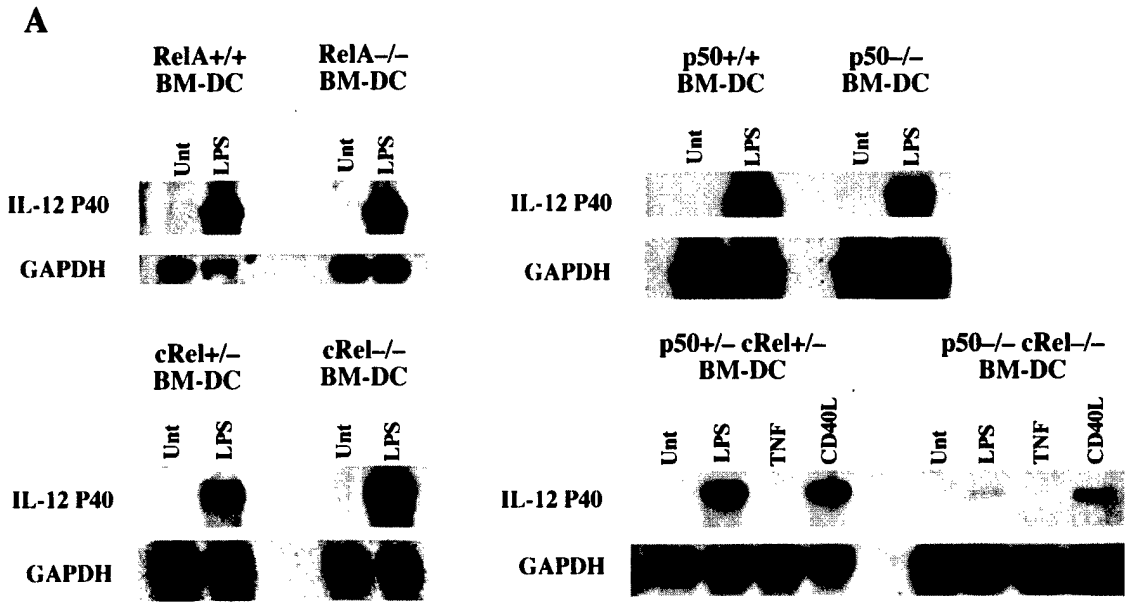
Figure 5. Analysis of $p50^{-/-}cRel^{-/-}$ DCs

(A) Immunostaining of spleen-derived DCs from $p50^{+/+}cRel^{+/+}$ and $p50^{-/-}cRel^{-/-}$ mice. Spleen DC were isolated from $p50^{+/+}cRel^{+/+}$ and $p50^{-/-}cRel^{-/-}$ mice. DCs were left unstained or stained with FITC-conjugated antibody against murine CD8α and with PE-conjugated antibody against CD11c for analysis by FACS. $CD11c^{+}CD8\alpha^{-}$ cells are shown in the left rectangle while $CD11c^{+}CD8\alpha^{+}$ are shown in the right rectangle, along with respective percentages.

(B) Gel shift analysis of nuclear extracts from $p50^{-/-}$, $cRel^{-/-}$, and $p50^{-/-}cRel^{-/-}$ bone marrow-derived DCs. Cells were left untreated or stimulated for 30 min and 2 hr with LPS and nuclear extracts prepared. NF-κB activation was analyzed by EMSA.

(C) Immunostaining of bone marrow-derived DCs from $p50^{+/+}cRel^{+/+}$ and $p50^{-/-}cRel^{-/-}$ mice. In the top panel, unstained cells are shown by the shaded histogram and $CD11c^{+}$ cells are shown by the open histogram. In other panels, BM-DCs were left untreated (shaded histogram) or were stimulated for 24 hr with LPS or CD40L (open histograms). Cells were left unstained or stained with FITC-conjugated antibodies against H2-K, I-A, and B7.2, and with PE-conjugated antibodies against CD11c, B7.1, and ICAM-1 for FACS analysis.

(D) Allogeneic MLR mediated by $cRel^{+/+}p50^{+/+}$and $cRel^{-/-}p50^{-/-}$ bone marrow-derived DCs. $CD3^{+}$ T cells (100,000) from BALB/c were incubated with γ-irradiated bone marrow dendritic cells (500 or 10,000) from wild-type and $p50^{-/-}cRel^{-/-}$ mice. DCs were left untreated or treated with LPS for 16 hr as indicated. After 3 days of culture, ^{3}H-thymidine incorporation was determined.



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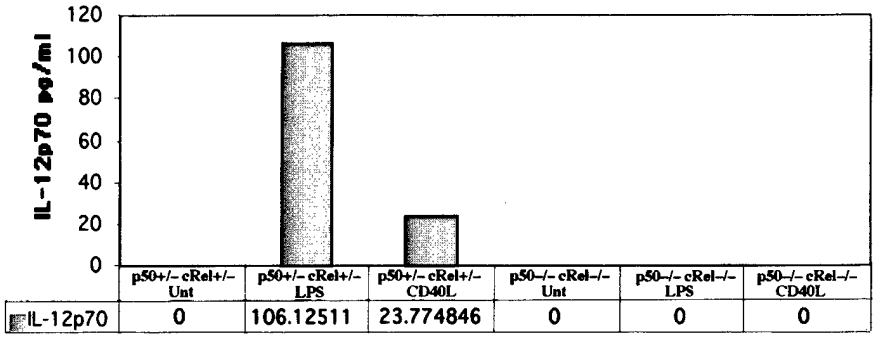

	p50+/– cRel+/– Unt	p50+/– cRel+/– LPS	p50+/– cRel+/– CD40L	p50–/– cRel–/– Unt	p50–/– cRel–/– LPS	p50–/– cRel–/– CD40L
IL-12p70	0	106.12511	23.774846	0	0	0

Figure 6. Analysis of IL-12 Expression in DCs

(A) Analysis of IL-12 p40 mRNA expression in bone marrow-derived DCs from RelA-, p50-, cRel-, and p50+cRel-deficient mice. Cells were left untreated or stimulated for 16 hr with LPS or CD40L. RNA was extracted, and IL-12 p40 mRNA expression was analyzed by Northern blotting. RNA loading was controlled by probing with a specific probe for murine GAPDH.

(B) Analysis of IL-12 p70 production by $p50^{+/-}cRel^{+/-}$ and $p50^{-/-}cRel^{-/-}$ bone marrow-derived DCs. Cells were left untreated or stimulated for 16 hr with LPS or CD40L. Culture supernatants were collected, and IL-12 p70 production was qualified by ELISA.

comprehensive analysis of the function of immunomodulatory NF-κB transcription factors in DCs. Foremost, our results have identified an essential and nonredundant role for NF-κB proteins in regulating development and survival of DCs. The implications of the findings reported here in regulation of DC development and function are discussed below.

Control of DC Development by NF-κB Proteins

Mature DCs were found to have high inducible levels of nuclear NF-κB complexes containing p50, cRel, and to a lesser extent, RelA. However, the individual absence of any of these proteins resulted in no obvious defects in DC survival or maturation. In contrast, absence of p50+RelA or p50+cRel resulted in significantly impaired development and survival/IL-12 production in DCs, respectively. These results therefore indicate redundant functions for p50 and RelA in developing DCs and p50 and cRel in mature DCs. These results also suggest that p50 and RelA may be the major NF-κB subunits in developing DCs, while p50 and cRel are the predominant subunits in mature DCs (as shown in Figures 1 and 5). Nonetheless, our results do not indicate exclusive roles for RelA, p50, and cRel in developing or mature DCs. Indeed, it is possible that these subunits interact and participate with p52 and RelB in controlling DC develop-

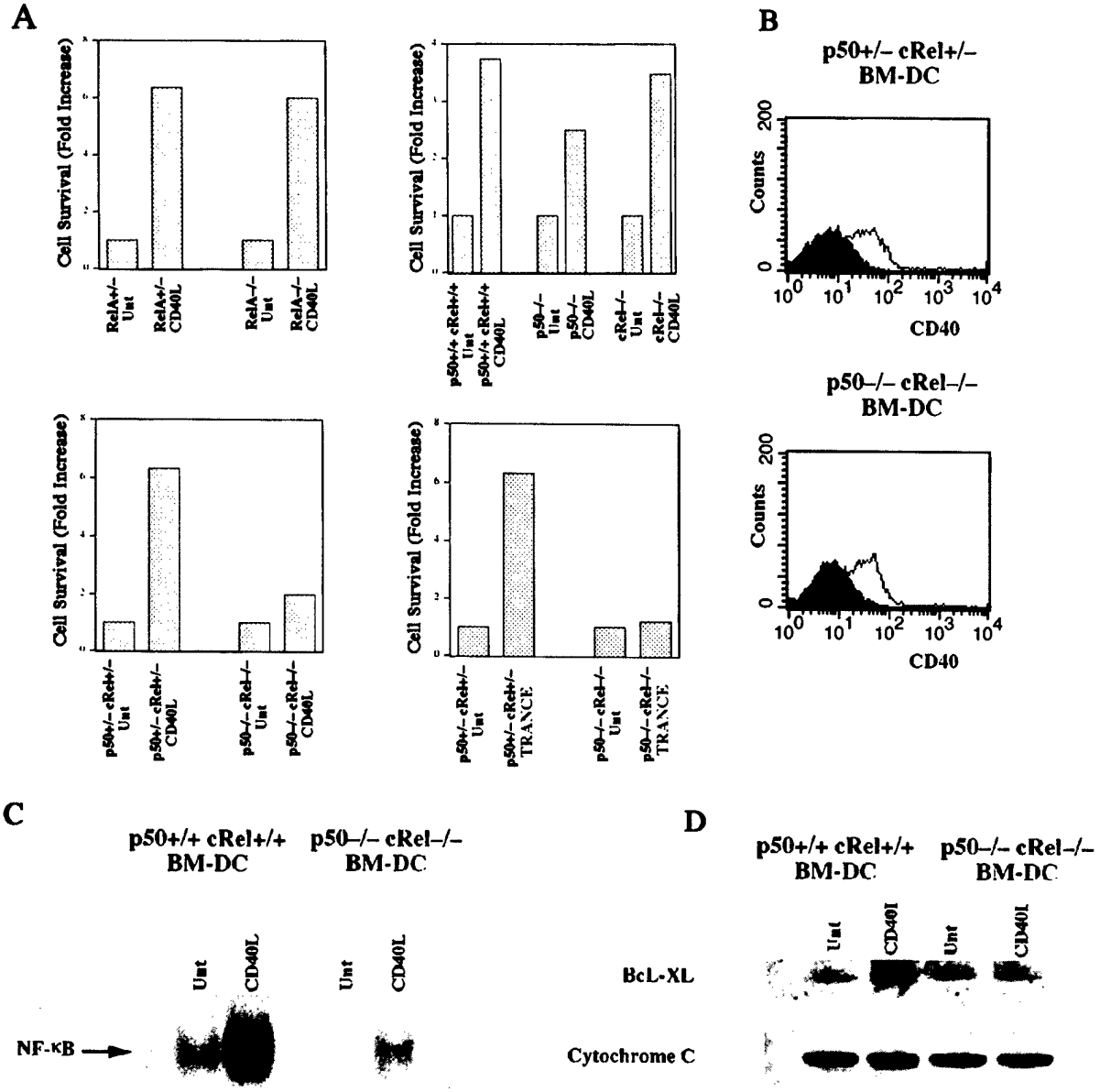

Figure 7. Regulation of DC Survival by CD40L and TRANCE

(A) CD40L- and TRANCE-induced DC survival analysis in RelA-, p50-, cRel-, and p50+cRel-deficient mice. BM-DCs were left untreated or stimulated with recombinant CD40L or TRANCE for 5 days. Cell viability was assayed by Trypan blue exclusion (CD40L) or propidium iodide staining (TRANCE). CD40L- and TRANCE-induced DC survival is expressed as a fold increase of survival compared to untreated cells.

(B) CD40 cell surface expression on $p50^{+/+}cRel^{+/+}$ and $p50^{-/-}cRel^{-/-}$ bone marrow-derived DCs. Cells were left unstained or stained with FITC-conjugated antibody against CD40 and analyzed by FACS.

(C) Gel shift analysis of nuclear extracts from $p50^{+/+}cRel^{+/+}$ and $p50^{-/-}cRel^{-/-}$ bone marrow-derived DCs. Cells were left untreated or stimulated for 36 hr with CD40L and nuclear extracts prepared. NF-κB activation was analyzed by EMSA.

(D) Analysis of BcL-xL protein expression in $p50^{+/+}cRel^{+/+}$ and $p50^{-/-}cRel^{-/-}$ bone marrow-derived DCs. Cells were left untreated or stimulated for 36 hr with recombinant CD40L and cytosolic extracts prepared. BcL-xL expression was analyzed by Western blot. Anti-Cytochrome C antibody was used to control protein loading.

ment and specific aspects of DC function. Interestingly, $p50^{-/-}cRel^{-/-}$ $CD4^+$ T cells have significantly impaired proliferative responses, indicating that these proteins may play a key role in multiple cell types of the immune system (Zheng et al., 2001). Even though deficiency in specific subunits is expected to affect all complexes containing these subunits, defects in DCs lacking p50+RelA or p50+cRel may in large part be due to complete absence of p50+RelA or p50+cRel heterodimeric complexes.

The combined absence of p50+RelA dramatically impaired DC development, as evidenced by a virtually complete lack of spleen $CD11c^+$ DCs (and thus both $CD11c^+CD8\alpha^-$ and $CD11c^+CD8\alpha^+$) and absence of cells exhibiting DC morphology. However, in this study we have not examined Langerhan's cell generation, which

is thought to represent a DC lineage distinct from both myeloid or lymphoid DCs. Significantly, our results have demonstrated normal generation of $p50^{-/-}RelA^{-/-}$ monocytic/macrophage lineage in vivo. In addition, studies of cotransplanted mice showed normal $p50^{-/-}RelA^{-/-}$ lymphopoiesis, while DC generation remained impaired. We believe these results are significant because they demonstrate that p50+RelA are not essential for generation of myeloid or lymphocyte precursors capable of differentiating into macrophages, lymphocytes, or DCs. Instead, our results indicate that p50+RelA are specifically required during differentiation of these precursors into myeloid- or lymphoid-derived DCs.

To provide insights into potential mechanisms responsible for impaired DC generation, we derived myeloid DCs from BM of $p50^{-/-}RelA^{-/-}$ FL-transplanted mice. In comparison to control cultures, the yield of $p50^{-/-}RelA^{-/-}$ DCs was considerably reduced. Our results indicate that the low yields are likely the consequence of cell death and may indicate: (1) high susceptibility of common myeloid precursors of DCs and macrophages to cell death; (2) inability of $p50^{-/-}RelA^{-/-}$ myeloid precursors to differentiate into DCs and which therefore undergo passive cell death in culture; and (3) cell death of $p50^{-/-}RelA^{-/-}$ precursors during differentiation into DCs. The first possibility appears unlikely since normal numbers of macrophages could be generated from $p50^{-/-}RelA^{-/-}$ myeloid precursors. Instead, the second and third possibilities are more consistent with our data. Although these two possibilities are difficult to discriminate at present, the generation of some DCs suggests that p50+RelA are not absolutely required for commitment to the DC lineage and thus are more likely required for survival of developing DCs. A key unresolved question is the mechanism of activation of p50+RelA complexes; e.g., are they specifically activated by exogenous ligands acting on developing DCs or by cell-intrinsic mechanisms that are induced during DC development? Our results also indicate that absence of myeloid-related DCs in $RelB^{-/-}$ mice may reflect a specific requirement for RelB in survival of the myeloid DC subset (Wu et al., 1998). Studies of mice deficient in the transcription factors Ikaros and PU.1 have identified defects in development of multiple lineages (Cortes et al., 1999; McKercher et al., 1996), including DCs (Anderson et al., 1998; Wu et al., 1997). In contrast, our results and studies of RelB mice indicate a much more specific function for NF-κB in DC development. Nonetheless, the precise roles played by Ikaros, PU.1, and NF-κB proteins in DC development remain to be determined.

Control of DC Survival by NF-κB Proteins

The high susceptibility of DCs to apoptosis has been well documented by in vitro and in vivo studies. However, during an antigen-specific immune response, DCs that are able to interact with T cells expressing CD40L and TRANCE are protected from cell death. Enhancement of DC longevity in this manner can profoundly impact T cell priming, but mechanisms involved in this process are poorly understood. In contrast to the absence of p50+RelA, we have found that DCs are generated normally in the absence of p50+cRel subunits but that these proteins are essential for regulating CD40L- and TRANCE-induced survival of mature DCs. Our results also indicate that impaired Bcl-xL induction following CD40L treatment of $p50^{-/-}cRel^{-/-}$ may be responsible for their reduced survival. These findings have thus uncovered an essential role for NF-κB proteins in CD40L- and TRANCE-induced survival pathways in DCs.

DC Maturation in NF-κB-Deficient DCs

DC maturation accompanies distinct changes in morphology and in expression of immunostimulatory molecules. Promoter studies have shown an important role for NF-κB sites in regulating expression of MHC and costimulatory molecules, in particular H-2K, B7-1, B7-2, and ICAM-1 (Baeuerle and Henkel, 1994; Ghosh et al., 1998). Our studies in fibroblasts have also identified essential roles for NF-κB proteins in regulating cell surface expression of H-2K and ICAM-1 (our unpublished data). In DCs, high NF-κB levels have been detected (Granelli-Piperno et al., 1995), suggesting their possible involvement in MHC and costimulatory molecule expression. Finally, treatment of DCs with the antioxidant NAC and the protease inhibitor TPCK, which inhibit NF-κB activation but also have additional pleiotropic effects, has been used as evidence for a role of NF-κB in regulating T cell stimulatory molecule expression in DCs (Rescigno et al., 1998; Verhasselt et al., 1999).

However, we have found that DCs lacking p50, RelA, or cRel exhibited no obvious defects in expression of MHC I, II, or the costimulatory molecules B7-1, B7-2, and ICAM-1. Most surprising were results on $p50^{-/-}cRel^{-/-}$ DCs, which showed dramatically reduced κB site binding activity but apparently normal LPS- and CD40L-induced upregulation of MHC and costimulatory molecules. It is nonetheless possible that additional κB site binding activity comprising of p52 and/or RelB, which may not be readily detectable by EMSA, can function in DC maturation. In addition to LPS, DC maturation can also be induced by several additional microbial agents including PGN, CpG, and dsRNA (Banchereau et al., 2000). It will therefore be important to determine whether maturation induced by these agents is dependent or independent of NF-κB proteins.

We have, however, found that NF-κB proteins are essential for LPS- and CD40L-induced IL-12 expression. Thus, $p50^{-/-}cRel^{-/-}$ DCs were found completely impaired in inducing IL-12 expression, while $p50^{-/-}$, $cRel^{-/-}$, or $RelA^{-/-}$ showed normal expression. In contrast to an essential and highly specific role of cRel in regulating p40 IL-12 expression in macrophages (Sanjabi et al., 2000), our results show that p40 expression is dependent on redundant functions of p50 and cRel in DCs. Interestingly, cRel was recently shown to be essential for IL-12 p35 expression in $CD8\alpha^+$ (Grumont et al., 2001) but dispensable for IL-12 p40 expression (consistent with the findings reported here). These results thus indicate further specificity of NF-κB subunit function in different DC subsets. Together with previous studies on $IRF\text{-}1^{-/-}$ mice (Lohoff et al., 1997; Taki et al., 1997), our findings demonstrate that NF-κB and IRF-1 factors may play the most essential roles in regulating IL-12 expression. Taken together, our results indicate that NF-κB proteins appear essential for the final phase

of DC "activation" triggered by interaction with T cell-expressed CD40L and TRANCE molecules, and resulting in enhancement of DC survival and IL-12 production.

In conclusion, our results have demonstrated highly specific functions for NF-κB transcription factors in DCs. Our results show that DC development and survival/IL-12 expression are regulated by NF-κB proteins in a highly subunit-specific manner. The key role played by DCs in regulating T cell activation and tolerance has generated considerable interest in the therapeutic potential of DCs. The apparent independence of DC maturation and survival may thus allow development of therapeutic strategies that can individually target these pathways in DCs.

Experimental Procedures

Mice

C57BL/6 CD45.2(Ly5.1), C57BL/6 CD45.1 (Ly5.2) congenic mice were obtained from NCI (Bethesda, MD). BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, ME. The cRel$^{-/-}$ (Kontgen et al., 1995), p50$^{-/-}$ (Sha et al., 1995), and RelA$^{-/-}$ (Beg et al., 1995) mice were interbred to generate p50$^{-/-}$cRel$^{-/-}$ and p50$^{-/-}$RelA$^{-/-}$ mice. All experiments were performed in accordance with institutional guidelines.

Adoptive Transfer of Fetal Liver Cells

Adoptive transfers were performed as previously described (Zheng et al., 2001). Donor and recipient cells were discriminated by staining with mAbs against two isoforms of CD45 (common leukocyte antigen), CD45.1 for recipient cells and CD45.2 for donor cells. For coinjection experiments described in Figure 4, 75% fetal liver cells (CD45.2) were mixed with 25% CD45.1 bone marrow cells and injected as above.

DC Preparation and Treatment

Bone Marrow-Derived and Spleen Dendritic Cells

Bone marrow-derived dendritic cells (BM-DCs) were made from BM suspensions prepared from femurs and tibias (Inaba et al., 1992) of control and NF-κB-deficient mice. Cells were cultured in 24-well plates in RPMI-1640 (GIBCO-BRL) supplemented with 5% fetal bovine serum, antibiotics, and 2-ME in the presence of purified mouse rGM-CSF (R&D systems) or supernatant (3% vol/vol) from J558L cells transduced with murine GM-CSF. For certain experiments (e.g., EMSA, Northern blotting), DC cultures were supplemented with IL-4 in order to enhance DC yield. Dendritic cell clusters were harvested after 6 days of culture by transferring to new culture plates. To obtain macrophages, bone marrow cells were cultured with 10 ng/ml of rM-CSF for 6 days. DCs were stimulated in the presence of 5 μg/ml of LPS (Sigma), 10 ng/ml of TNF-α (R&D systems), 1 μg/ml of purified recombinant TRANCE, or 1/100 dilutions of recombinant CD40L-containing baculovirus supernatant (Wong et al., 1997) for the time periods indicated in the text. Spleen dendritic cells were prepared from collagenase D-digested spleens (Wong et al., 1997).

Flow Cytometry Analysis

Untreated or LPS/CD40L-treated DCs were harvested at the time periods indicated in the text. Prior to immunostaining with labeled antibodies, DCs were incubated with Fc-γ block antibody (anti-mouse CD16/CD32) to avoid the nonspecific binding of Abs to Fc-γ receptors. The cells were then stained with FITC- or PE-conjugated anti-mouse mAbs for MHC-1 (H-2K), MHC-II (I-A), CD80, CD86, ICAM-1, CD40, CD11c, CD11b, and B220 (Pharmingen) for 30 min on ice. Cells were then washed twice, fixed in 4% paraformaldehyde, and analyzed using a Becton Dickinson flow cytometer.

Northern, Western, and EMSA Analysis

Northern blotting was performed as described previously (Ouaaz et al., 1999) by using specific radio-labeled cDNA probes for IL-12 p40 and GAPDH genes. Western analysis was carried out with anti-Bcl-x_L or anti-Cytochrome C Abs (Santa Crutz Biotechnology). Nuclear and cytoplasmic extract preparation and EMSA were carried out using a previously described procedure (Zheng et al., 2001). In some experiments, nuclear proteins were preincubated with anti-RelA (p65), anti-p50, and anti-cRel Abs (Santa Crutz Biotechnology) for 15 min prior to incubation with the κB site oligonucleotide probe.

Allogeneic MLR

$CD3^+$ T cells from BALB/c mice (haplotype "*d*") were cocultured with γ-irradiated (3000 rads) bone marrow or spleen dendritic cells from C57BL/6-L129 NF-κB-deficient mice (haplotype "*b*") in RPMI (GIBCO-BRL) medium with 2-ME supplemented with 10% fetal bovine serum and antibiotics. After 3 days, the cultures were pulsed with 1 μCi/well (^{3}H)-thymidine for the last 16 hr, harvested, and counted using a Micro-Betaplate reader. All assays were performed in triplicate, and standard deviations are shown in the figures.

DC Survival Assays

On day 6, BM-DC clusters were dislodged, washed, and transferred to new culture dishes and incubated overnight. On day 7, DCs were left untreated or treated with 1 μg/ml of purified recombinant TRANCE or 1/100 dilutions of recombinant CD40L-containing baculovirus supernatant (Wong et al., 1997). DC survival was quantified by measuring cell viability (Trypan blue exclusion or PI staining) as shown by fold increase in cell survival compared to untreated DCs.

Acknowledgments

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Combined Deficiency of p50 and cRel in CD4$^+$ T Cells Reveals an Essential Requirement for Nuclear Factor κB in Regulating Mature T Cell Survival and In Vivo Function

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Abstract

Signaling pathways involved in regulating T cell proliferation and survival are not well understood. Here we have investigated a possible role of the nuclear factor (NF)-κB pathway in regulating mature T cell function by using CD4$^+$ T cells from p50$^{-/-}$ cRel$^{-/-}$ mice, which exhibit virtually no inducible κB site binding activity. Studies with these mice indicate an essential role of T cell receptor (TCR)-induced NF-κB in regulating interleukin (IL)-2 expression, cell cycle entry, and survival of T cells. Our results further indicate that NF-κB regulates TCR-induced expression of antiapoptotic Bcl-2 family members. Strikingly, retroviral transduction of CD4$^+$ T cells with the NF-κB–inducing IκB kinase β showed that NF-κB activation is not only necessary but also sufficient for T cell survival. In contrast, our results indicate a lack of involvement of NF-κB in both IL-2 and Akt-induced survival pathways. In vivo, p50$^{-/-}$ cRel$^{-/-}$ mice showed impaired superantigen-induced T cell responses as well as decreased numbers of effector/memory and regulatory CD4$^+$ T cells. These findings provide the first demonstration of a role for NF-κB proteins in regulating T cell function in vivo and establish a critically important function of NF-κB in TCR-induced regulation of survival.

Key words: T lymphocytes • T cell receptor • cell death • NF-κB • transcription factor

Introduction

Activation of CD4$^+$ Th cells is a critical step in initiating an adaptive immune response. Two signals, both of which are delivered by APCs, are required for activation-induced proliferation of Th cells: (a) engagement of the TCR by an antigen–MHC complex on APCs, and (b) engagement of costimulatory molecules, the best characterized of which is CD28, with the B7 family of proteins expressed by APCs. TCR and CD28-induced pathways synergize in stimulating T cell proliferation and in regulating expression of growth promoting cytokines, such as IL-2 (1–3). Significantly, T cell responses are also intimately dependent upon survival-promoting signals induced by TCR, CD28, and cytokine receptors.

Antigen encounter by T cells induces both proliferative and survival pathways, which drive T cell expansion and lead to the development of immunity. Conversely, after antigen is cleared, cessation of T cell–APC engagement can result in rapid induction of cell death (4, 5). This mode of T cell death, referred to as "passive" cell death, is thought to be crucial for terminating an immune response and maintaining T cell homeostasis (5). The best-defined survival pathways in T cells involve growth-promoting cytokines, such as IL-2, or costimulatory molecules, such as CD28, that appear to function by activating the antiapoptotic kinase, Akt (see below). TCR engagement itself also provides protection through a signaling pathway that has not yet been defined. In contrast, when T cells encounter high concentrations of antigens (e.g., autoantigens), TCR signals promote apoptosis either by triggering death receptors such as Fas or TNFR1 (4–6), or by activating the proapoptotic molecule Bim (7). This form of T cell death is referred to as "activation-induced" cell death and helps prevent autoimmunity. Thus, regulation of T cell responses is dependent upon both antiapoptotic and proapoptotic signaling pathways.

T cell activation is initiated by protein tyrosine kinases, which induce the activation of multiple signaling pathways (8). Several transcription factors have been shown to be activated by TCR, CD28, and cytokine receptor engage-

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ment, including those belonging to the Ets, NFAT, AP-1, nuclear factor (NF)*-κB, and signal transducer and activator of transcription families (9). However, the specific roles played by these factors in regulating T cell activation and survival is only beginning to be understood. Gene targeting studies have recently shed some light on the function of these transcription factors in T cells. Studies of mature T cells singly or doubly deficient in NFAT proteins (10) have primarily revealed defects in T cell differentiation rather than activation or survival (11–13). c-Jun$^{-/-}$ T cells also show no proliferative defects (14) whereas the role of other AP-1 family proteins in T cells is still unclear. Recent studies have indicated that the PI3K/Akt pathway is a key survival mediator of both CD28 and IL-2R (15–18). However, it is not known how the Akt protein kinase blocks apoptosis in T cells, although NF-κB is thought to be one of the targets of Akt (18–21).

The NF-κB transcription factors are key regulators of inflammatory and immune response genes (22). NF-κB activation is controlled by signal-dependent phosphorylation of NF-κB–associating IκB inhibitory proteins by IκB kinases (IKKα and IKKβ; reference 23). Phosphorylated IκB proteins are rapidly degraded, allowing translocation of NF-κB to the nucleus. Recent studies have shown an important role for NF-κB proteins in regulating cell death in many different cell types (22), including B cells (24–26) and dendritic cells (DCs; reference 27). In T cells, NF-κB is activated by both TCR and CD28 engagement and comprises of p50+RelA and p50+cRel heterodimers and p50 homodimers (28). However, in vitro proliferative responses in p50$^{-/-}$, RelA$^{-/-}$, and cRel$^{-/-}$ CD4$^+$ T cells are only moderately reduced compared with WT T cells (28). In contrast, proliferation of p50$^{-/-}$ cRel$^{-/-}$ doubly deficient T cells is drastically reduced, indicating important but redundant functions for p50 and cRel in T cells (28). NF-κB function in T cells has also been studied in transgenic (Tg) mice expressing a degradation-resistant form of the IκBα inhibitor in T cells (29–31). Studies of IκB-Tg T cells also showed impaired in vitro proliferative responses (29). Gene targeting studies of PKCθ and Bcl-10 have revealed both impaired NF-κB activation and T cell proliferative responses in vitro (32, 33). However, impaired proliferation may result from defects in cell cycle regulation and/or decreased survival of activated T cells. Studies to date have not discriminated between these different possible functions for NF-κB in T cells. Significantly, the possible function of NF-κB in regulating TCR, CD28, and cytokine receptor–induced pathways has not been determined. Finally, in vitro studies of T cell function may not accurately reflect in vivo events. At present, the in vivo function of NF-κB in T cells remains to be addressed.

Here we have examined the function of NF-κB in regulating mature T cell signaling pathways by using p50$^{-/-}$ cRel$^{-/-}$ CD4$^+$ T cells, which have virtually no inducible NF-κB activity. We show that NF-κB plays essential roles in regulating TCR-induced cell cycle entry and survival both in vitro and in vivo. In contrast, NF-κB is not necessary for survival pathways induced by Akt or IL-2. Our findings define NF-κB as a key participant in the TCR-induced survival pathway that is not only essential but also sufficient for maintaining T cell survival.

Materials and Methods

Isolation, Activation, and Cell Division Analysis of CD4$^+$ T Cells

p50$^{-/-}$ cRel$^{-/-}$ mice were generated by crossing p50$^{-/-}$ mice (34) with cRel$^{-/-}$ mice (provided by S. Gerondakis, The Walter and Eliza Hall Institute of Medical Research, The Royal Melbourne Hospital, Victoria, Australia; reference 35). OTII×IL-2$^{-/-}$ mice were generated by crossing OT-II mice (36) with IL-2$^{-/-}$ mice (37). All mice were used between 2 to 4 mo after birth. CD4$^+$ T cells were isolated from mouse spleens using CD4$^+$ Dynabeads (Dynal) according to the manufacturer's instructions. Isolated T cells were cultured in T cell medium (RPMI containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, and 0.1 mM 2-mercaptoethanol; GIBCO BRL). T cells were activated by culture in the presence of 1 μg/ml plate-bound αCD3 and 1 μg/ml αCD28 (BD Biosciences) monoclonal antibodies. In some experiments, 20 ng/ml murine IL-2 (R&D Systems) was also added to T cell culture media. For proliferation analysis, naive CD4$^+$ T cells were incubated in T cell media containing 1 μM 5-chloromethylfluorescein diacetate (Molecular Probes) at 37°C for 30 min, washed twice, and cultured under different activation conditions for 1–3 d before FACS® analysis. 5-chloromethylfluorescein diacetate breaks into carboxyfluorescein diacetate succinimidyl ester (CFSE) in cells and then the latter covalently associates with cellular proteins. When a cell divides, CFSE gets evenly distributed in two cells. Each cell division causes a "left shift" in the FACS® histograph of ~0.3 log scale units. CFSE analysis was performed on living cells after gating on them based on their forward and side scatter characteristics.

FACS®, Electrophoretic Mobility Shift Assay (EMSA), RT-PCR, and Northern Analysis

PE-αCD4, PerCP-αCD4, FITC-αCD8, PE-αB220, FITC-αCD3, FITC-αCD25, PE-αCD25, FITC-αTCRVβ6, FITC-αTCRVβ8, FITC-αCD44, and PE-αCD62L antibodies were purchased from BD Biosciences. FACS® analysis was performed on a FACSCalibur® cytometer (Becton Dickinson). EMSAs were performed as previously described (28). The hairpin oligonucleotide probes used were GAGAGGGGATTCCCCGATTACCTTTCGGGGAATCCCCTCT (H2 site) and GAGAGGGGAATCTCCCATTAGCTTTGGGAGATTCCCCTCT (IL-2Rα site). Total RNA of T cells stimulated under different conditions were extracted using TRI reagent (Molecular Research Center) as recommended by the manufacturer. RT was performed using PRO-Star RT-PCR First Strand Kit from Stratagene according to the manufacturer's instructions. The primers used in the experiments were: IL-2: 5′ primer AACAGCGCACCCACTTCAA, 3′ primer TTGAGATGATGCTTTGACA; c-Myc: 5′ primer CGACGATGCCCCTCAACGTG, 3′ primer

*Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; NF, nuclear factor; PI, propidium iodide; SEB, staphylococcal enterotoxin B; Tg, transgenic.

CGAGTTAGGTCAGTTTATGC; Bcl2: 5′ primer GCTTC-TTTTCGGGGAAGGAT, 3′ primer AAGCCCAGACTCAT-TCAACC; Bcl-X_L: 5′ primer AAG CAA GCG CTG AGA GAG GCA, 3′ primer ACA GTC ATG CCC GTC AGG AAC; and β actin: 5′ primer ATGGATGACGATATCGCT, 3′ primer ATGAGGTAGTCTGTCAGGT. Northern blotting was performed as previously described (38), using specific radiolabeled cDNA probes for Bcl2, Bcl-X_L, and β actin genes.

Staphylococcal Enterotoxin B (SEB) Challenge Experiments

WT and $p50^{-/-}$ $cRel^{-/-}$ mice were injected intravenously with 50 μg SEB (Toxin Technology). Mice were killed before injection (day 0) and 3 and 6 d after injection. Splenocytes were double stained with CD4 and TCR-Vβ8 or TCR-Vβ6 antibodies to determine the percentage of $V\beta8^+$ or $V\beta6^+$ T cells in total $CD4^+$ T cell population.

Retroviral Infection of $CD4^+$ T Cells and Infected Cell Survival Assays

The constitutively active IKKβ mutant, IKKβ(EM), which contains both EE and M10 mutations, was provided by M. Delhase and M. Karin (University of California San Diego, San Diego, CA; reference 39). It was subcloned into murine stem cell virus internal ribosome entry site green fluorescent protein (GFP; MIG) retroviral expression vector (16). MIG-Bcl2 and MIG-MyrAKT constructs have been described (15, 16). Retroviruses were produced by transfecting BOSC 293T cells (40) with retroviral plasmid DNA and the pCL-Eco packaging plasmid.

During infection, WT and $p50^{-/-}$ $cRel^{-/-}$ T cells were activated with 1 μg/ml αCD3 and 1 μg/ml αCD28 in the presence of 20 ng/ml IL-2. OTII T cells were activated with 1 μg/ml OVA peptide in the presence of 10 ng/ml IL-2. $CD4^+$ T cells were spin infected at 2,500 rpm for 1 h at 30°C on days 1 and 2 of T cell activation. Viable cells were harvested on day 4 by Ficoll (Amersham Biosciences) gradient centrifugation. Because all retroviral constructs based on MIG vector contain an internal ribosome entry site GFP cassette, infected cells were distinguished from uninfected cells by GFP expression using FACS®. T cell survival assays were performed as follows: after infection, viable T cells were obtained by Ficoll spin. Then, the percentage of GFP^+ cells was determined by FACS®. This was the starting time point T_0 (day 0). These T cells were plated in T cell medium without αCD3 antibody. At different time points T_n (days 1, 2, and 3), cells were stained with 2.5 μg/ml propidium iodide (PI) for 10 min before FACS® analysis. Viable infected T cells were represented by the GFP^+ PI^- population. The survival rate of the infected T cells was calculated according to this formula: (the percentage of GFP^+ PI^- cells at T_n)/(the percentage of GFP^+ PI^- cells at T_0) × 100.

Cell Death Assays

DNA Content Staining. DNA content staining was performed as previously described (28). In brief, T cells were fixed at 4°C in 70% ethanol for 24 h and stained with a PI staining solution (PBS containing 50 μg/ml PI, 100 U/ml RNase A, and 1 mg/ml glucose) for 2 h at room temperature before FACS® analysis. Apoptosis was determined by quantification of the sub-G_0 population.

Passive Cell Death Determination. After 3 d of activation, $CD4^+$ T cells were harvested by Ficoll (Amersham Biosciences) density gradient centrifugation. Typically, T cells obtained by this method contained <5% dead cells. These T cells were plated in T cell medium, with or without 20 ng/ml IL-2 for 1 or 2 d before being assayed for apoptosis by DNA content staining and quantification of the sub-G_0 population. In certain experiments, SYTOX was used to stain the apoptotic cell population. SYTOX (Molecular Probes) is a fluorescent dye that emits a strong green fluorescence after binding to DNA. Cells were double stained with PE-αCD25 and SYTOX (2 nM in PBS) before FACS® analysis. Apoptosis rate was calculated using this formula: (percentage of $CD25^+$ $SYTOX^+$ cells)/(percentage of total $CD25^+$ cells) × 100.

Cell Death of Retrovirus-infected T Cells. This is described above in the section on retrovirus infection.

Results

Normal T Cell Development in the Absence of p50 and cRel. Amongst known NF-κB subunits, p50, cRel, and RelA comprise a large proportion of NF-κB activity in mature T lymphocytes (28). Although T cell development is normal in $p50^{-/-}$, $cRel^{-/-}$, or $RelA^{-/-}$ mice, doubly deficient $p50^{-/-}$ $RelA^{-/-}$ and $IKK\beta^{-/-}$ mice lack lymphocytes, probably because of impaired survival of common lymphocyte precursors (34, 35, 41–44). Because p50+RelA and p50+cRel heterodimers comprise two major NF-κB complexes in many cell types, including T cells, we determined the consequence of the absence of p50 and cRel on lymphocyte development. $p50^{-/-}$ and $cRel^{-/-}$ mice (34, 35) were interbred to obtain $p50^{-/-}$ $cRel^{-/-}$ mice, which were fertile and apparently healthy. As shown in Fig. 1 A, no differences in the numbers of double-positive and CD4 and CD8 single-positive thymocytes were noticed between WT and $p50^{-/-}$ $cRel^{-/-}$ mice. Mature T and B cells were also present in normal numbers in the spleens of $p50^{-/-}$ $cRel^{-/-}$ mice, as were $CD4^+$ and $CD8^+$ T cells (Fig. 1 B). We have also found that DC development proceeds normally in these mice (27). Expression of the CD3 component of the TCR was indistinguishable between WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells (Fig. 1 C).

To determine the specific consequence of engagement of TCR and CD28 on NF-κB activation in WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells, we used plate-bound anti-CD3 (αCD3) and anti-CD28 (αCD28) monoclonal antibodies. EMSA analysis showed that in WT T cells, nuclear NF-κB activity binding with the high affinity H-2 or IL-2Rα (CD25) κB sites was significantly enhanced after a 6-h stimulation with αCD3 or αCD3+αCD28 (Fig. 1 D). The two indicated complexes have previously been characterized as heterodimers of p50 with RelA and cRel (complex 1) or homodimers of p50 (complex 2; reference 28). As expected from previous studies, αCD3-induced NF-κB activity could be enhanced in the presence of αCD28 (Fig. 1 D, bottom). Strikingly, both constitutive and αCD3-inducible κB site binding activities were virtually abolished in $p50^{-/-}$ $cRel^{-/-}$ T cells (Fig. 1 D). Furthermore, CD3+CD28 engagement did not enhance κB site binding activity in $p50^{-/-}$ $cRel^{-/-}$ T cells. Similar results were also obtained when T cells were used after 3 d of activation (unpublished data). These results demonstrate that

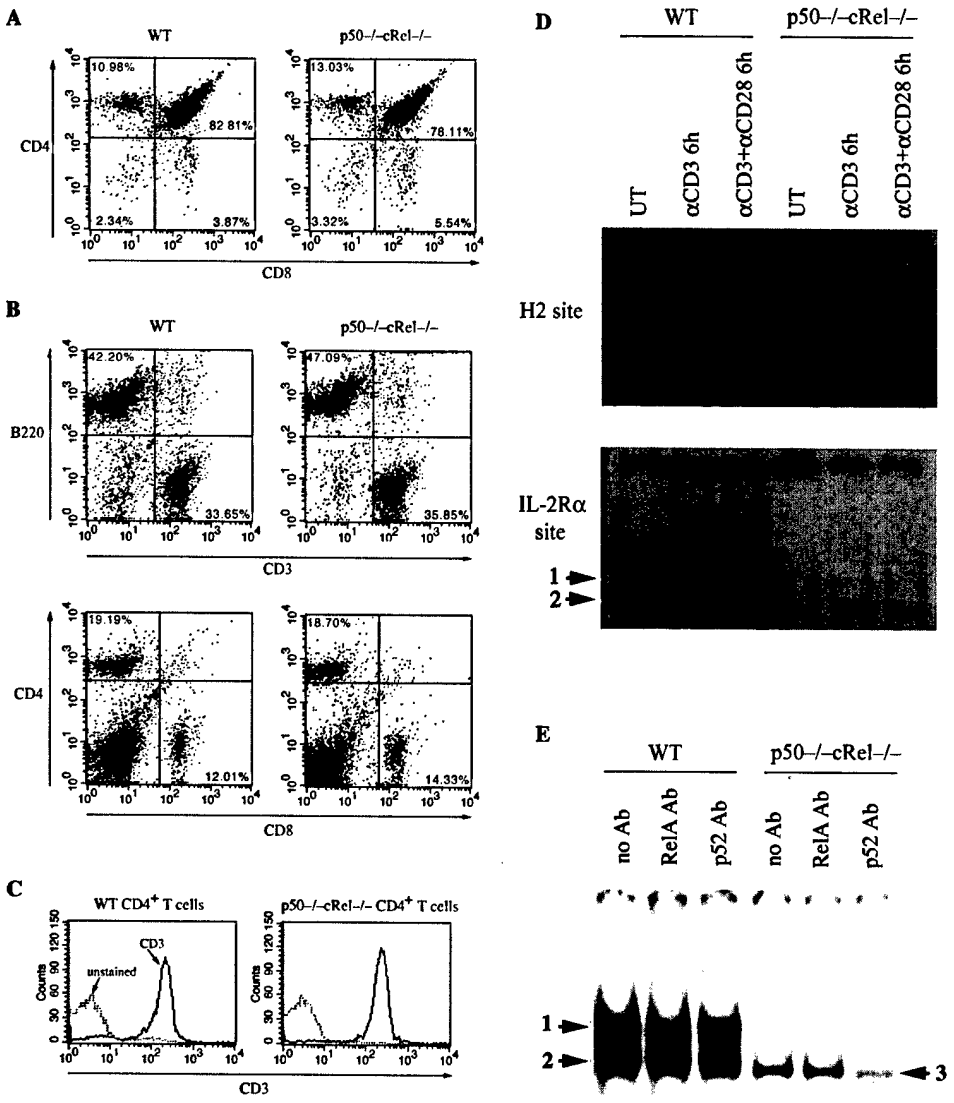

Figure 1. Combined absence of p50 and cRel NF-κB subunits does not affect T cell development. (A) FACS® analysis of CD4 and CD8 expression in WT and $p50^{-/-}$ $cRel^{-/-}$ thymocytes. (B) FACS® analysis of B220, CD3, CD4, and CD8 expression in WT and $p50^{-/-}$ $cRel^{-/-}$ splenocytes. (C) CD3 expression level in WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells. FACS® was performed by gating on $CD4^+$ cells. (D) EMSAs were performed with nuclear extracts from naive $CD4^+$ T cells either untreated or activated with 1 μg/ml plate-bound αCD3 or 1 μg/ml plate-bound αCD3 plus 1 μg/ml αCD28 for 6 h. NF-κB binding sites used were from H-$2K^b$ and IL-Rα (CD25). Complexes 1 and 2 are described in the text. (E) EMSAs were performed with nuclear extracts from naive $CD4^+$ T cells activated with 1 μg/ml plate-bound αCD3 plus 1 μg/ml αCD28 for 6 h. NF-κB binding site was from H-$2K^b$. The addition of antibodies to RelA and p100/p52 is indicated.

p50+cRel comprise a major proportion of NF-κB activity in mature $CD4^+$ T cells, as they do in mature B cells and DCs (27, 35, 45).

We then tested for the presence of RelA and p52, two NF-κB subunits that may substitute for p50 and cRel in $p50^{-/-}$ $cRel^{-/-}$ T cells. As previously shown (28) and in Fig. 1 E, the κB site binding activity in αCD3+αCD28–stimulated WT T cells is reduced by a RelA Ab (complex 1) and a p100/p52 Ab (complex 2). In contrast, the RelA Ab did not affect $p50^{-/-}$ $cRel^{-/-}$ T cell DNA binding activity although reactivity was evident with the p100/p52 Ab (complex 3). Importantly, RelA protein levels were no different between WT and $p50^{-/-}$ $cRel^{-/-}$ T cells (unpublished data). Thus, lack of RelA DNA binding in $p50^{-/-}$ $cRel^{-/-}$ T cells is not due to absence of RelA protein. Although it is unclear why RelA DNA binding is not detected in $p50^{-/-}$ $cRel^{-/-}$ T cells, a likely explanation might be that in the absence of p50 and cRel, RelA remains monomeric and/or forms homodimers. Although monomeric RelA cannot bind DNA, previous studies have shown that RelA homodimers poorly bind DNA compared with heterodimers with p50 (46), which may also explain why RelA is typically present in heterodimeric complexes with these proteins. Thus, the absence of p50 and cRel also diminishes the formation of RelA DNA binding complexes, resulting in further reduction in κB site binding activity. Overall, these results indicate that the normal development and greatly reduced κB site binding activity in $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells make them an excellent tool for studying NF-κB function in mature T cells.

Impaired Cell Cycle Entry after TCR Stimulation of $p50^{-/-}$ $cRel^{-/-}$ T Cells In Vitro. Using $p50^{-/-}$ $cRel^{-/-}$ cells, we first determined the role of NF-κB in cell cycle regulation of $CD4^+$ T cells. To this end, WT and $p50^{-/-}$ $cRel^{-/-}$ cells were stimulated with αCD3 for 2 d, after which DNA content per cell was quantified to simultaneously determine potential defects in regulation of cell cycle and cell survival. αCD3 treatment of WT T cells induced significant cell cycle entry, evidenced by S and G2/M phase cells (Fig. 2 A). In contrast, very few S and G2/M phase cells were de-

tected after αCD3 treatment of $p50^{-/-}$ $cRel^{-/-}$ T cells. Significantly, a large proportion of $p50^{-/-}$ $cRel^{-/-}$ cells underwent apoptosis as evidenced by the sub-G_0 population (Fig. 2 A). However, costimulation of $p50^{-/-}$ $cRel^{-/-}$ cells with αCD28 significantly enhanced cell cycle entry and reduced the number of apoptotic cells (Fig. 2 A), suggesting a potentially NF-κB–independent role for CD28 in regulating these processes. In contrast, the addition of exogenous IL-2 did not significantly affect T cell survival or cell cycle entry (Fig. 2 A). Next, we determined whether impaired cell cycle entry and survival of $p50^{-/-}$ $cRel^{-/-}$ cells was due to impaired expression of genes involved in the regulation of these processes. A 6-h treatment of WT T cells with αCD3 or αCD3+αCD28 increased the expression of mRNAs for IL-2, c-Myc, Bcl-2, and Bcl-xL (Fig. 2 B). On the other hand, similar treatment of $p50^{-/-}$ $cRel^{-/-}$ cells showed significantly impaired induction of IL-2 and Bcl-xL whereas induction of c-Myc and Bcl-2 was also moderately reduced. Thus, defects in cell cycle entry and/or survival of $p50^{-/-}$ $cRel^{-/-}$ T cells might be due to impaired induction of expression of these key genes after TCR engagement. Although our results do not demonstrate that NF-κB proteins directly regulate these genes, previous studies have identified κB sites in control regions of these genes (e.g., Bcl-xL; references 47 and 48), suggesting a possible direct role.

These results indicate potential defects in both cell cycle entry and survival of $p50^{-/-}$ $cRel^{-/-}$ T cells. To specifically determine whether $p50^{-/-}$ $cRel^{-/-}$ T cells exhibit a defect in cell cycle control, both WT and $p50^{-/-}$ $cRel^{-/-}$ cells were CFSE labeled and stimulated for 1–3 d with αCD3. At the end of stimulation, dead cells were gated out during FACS® and the level of CFSE was determined specifically in the viable cell population. The level of CFSE was used to determine the number of cell divisions that viable WT and $p50^{-/-}$ $cRel^{-/-}$ T cells underwent after stimulation. As shown in Fig. 3 A, either a 2- or 3-d αCD3 treatment led to multiple cell divisions in WT T cells whereas the vast majority of $p50^{-/-}$ $cRel^{-/-}$ did not undergo cell division. As shown above, IL-2 expression was significantly reduced in $p50^{-/-}$ $cRel^{-/-}$ cells. Therefore, we tested whether the addition of exogenous IL-2 could allow $p50^{-/-}$ $cRel^{-/-}$ T cell division. A 3-d treatment with αCD3+IL-2 moderately increased the number of dividing $p50^{-/-}$ $cRel^{-/-}$ cells (Fig. 3 B), but this number remained significantly lower than WT cells treated with αCD3 alone or with αCD3+IL-2. In contrast, CD28 costimulation was able to significantly increase the number of $p50^{-/-}$ $cRel^{-/-}$ cells that underwent cell division (consistent with the results shown in Fig. 2 A). As shown in Fig. 3 B, bottom right, a majority of WT cells stimulated with αCD3+CD28+IL-2 divided three to four times over a 3-d period. In contrast, the number of $p50^{-/-}$ $cRel^{-/-}$ cells that underwent more than two cell divisions when stimulated in the same manner was significantly reduced. Furthermore, most $p50^{-/-}$ $cRel^{-/-}$ cells failed to undergo even a single cell division. Significantly, $p50^{-/-}$ $cRel^{-/-}$ T cells stimulated with αCD3+CD28+IL-2 for 3 d expressed high levels of the IL-2Rα chain (CD25; Fig. 3 C), although less than in similarly treated WT cells. These results indicate that impaired cell cycle entry of $p50^{-/-}$ $cRel^{-/-}$ T cells is not due to lack of responsiveness to stimulation. Instead, $p50^{-/-}$ $cRel^{-/-}$ cells do undergo certain activation events, but fail to cycle. Therefore, these results demonstrate a crucial role for NF-

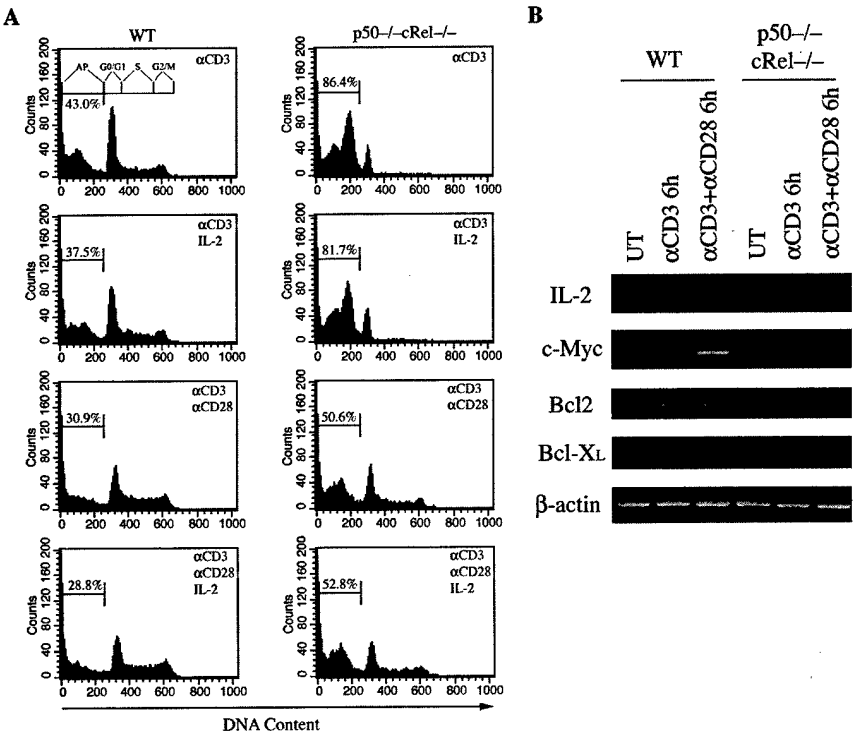

Figure 2. Impaired cell cycle entry and survival after TCR stimulation of $p50^{-/-}$ $cRel^{-/-}$ T cells. (A) WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells were activated with plate-bound αCD3, αCD3+IL-2, αCD3+αCD28, or αCD3+αCD28+IL-2 for 2 d before DNA content staining and FACS® were performed. The percentages show the sub-G_0 population, which represents apoptotic cells and cells in different phases of the cell cycle. Typical results of several independent experiments are shown. (B) RT-PCR was performed to determine expression of IL-2, c-Myc, Bcl2, and Bcl-X_L in WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells after 6 h of activation.

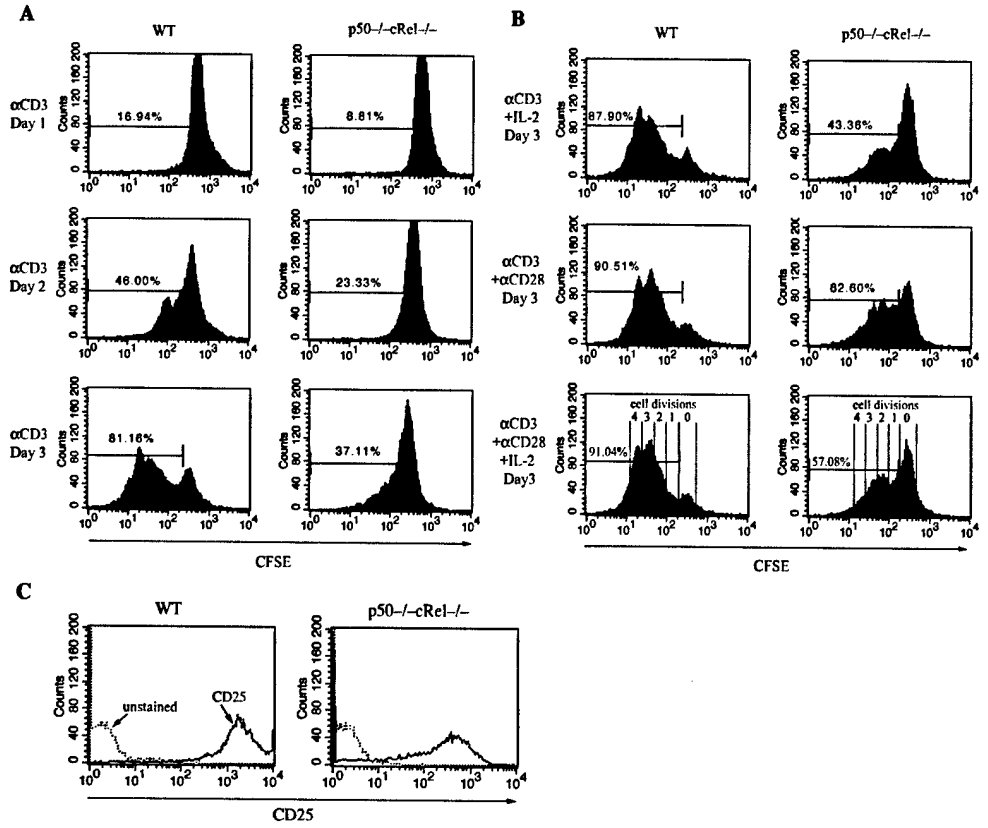

Figure 3. Impaired cell division of $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells after activation. (A and B) WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells were CFSE labeled and activated under different conditions as shown for 1–3 d. FACS® was performed on viable cells by gating on the forward and side scatter characteristics. As shown in the bottom right of B, peaks/shoulders represent the number of times cells underwent division. The percentage indicates cell population that has divided at least once. (C) FACS® analysis of CD25 (IL-2Rα) expression on WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells after 3 d of activation by plate-bound αCD3+αCD28 and IL-2.

κB proteins in regulating TCR-induced cell division. In addition, our results suggest that defects in cell division are not due to impaired IL-2 or IL-2R expression.

Increased Cell Death in Activated $p50^{-/-}$ $cRel^{-/-}$ T Cells Can Be Rescued by Bcl-2. The results shown above also indicate that the activation of $p50^{-/-}$ $cRel^{-/-}$ T cells render them more susceptible to cell death than WT T cells (Fig. 2 A). To test this possibility and the potential mechanisms involved, WT and $p50^{-/-}$ $cRel^{-/-}$ cells were stimulated with αCD3+αCD28 for 3 d, after which viable cells were isolated on a Ficoll gradient. These cells were then double labeled with αCD25 to detect activated T cells and the DNA dye SYTOX to detect apoptotic cells. This method was used to determine the percentage of activated T cells undergoing apoptosis (i.e., $CD25^+$ $SYTOX^+$) out of the total number of $CD25^+$ cells ($CD25^+$ $SYTOX^-$ and $CD25^+$ $SYTOX^+$). As shown in Fig. 4 A, immediately after activation (day 0), only 5% WT and $p50^{-/-}$ $cRel^{-/-}$ Ficoll-isolated cells were apoptotic. In the absence of stimulation, activated T cells rapidly undergo cell death. After 1 d in culture without stimulation, ~25% activated $CD25^+$ WT T cells underwent cell death. In contrast, 75% of $CD25^+$ $p50^{-/-}$ $cRel^{-/-}$ cells underwent cell death over the same period. Thus, activated $p50^{-/-}$ $cRel^{-/-}$ cells are significantly more susceptible to cell death than WT cells. Therefore, these results demonstrate that NF-κB proteins play an essential role in regulating not only cell division (Fig. 3), but also survival of activated T cells.

As shown in Fig. 2 B, expression of Bcl-2 was moderately reduced whereas expression of Bcl-xL was dramatically impaired in $p50^{-/-}$ $cRel^{-/-}$ T cells after TCR engagement. Importantly, it has been shown that Bcl-2 and Bcl-xL inhibit cell death by biochemically similar mechanisms (49). Therefore, we wanted to determine whether impaired survival of activated $p50^{-/-}$ $cRel^{-/-}$ cells was due to decreased expression of these Bcl-2 family members after TCR engagement. To this end, we first infected $p50^{-/-}$ $cRel^{-/-}$ T cells with a GFP-expressing polycistronic retrovirus (MIG; reference 16). All GFP^+ $p50^{-/-}$ $cRel^{-/-}$ cells were also $CD25^+$ because oncoretroviruses can only infect proliferating cells (Fig. 4 B; reference 50). WT and $p50^{-/-}$ $cRel^{-/-}$ T cells were then infected with MIG or MIG-Bcl-2 and incubated for 2 d without stimulation, after which the percentage of viable infected cells (GFP^+ PI^-) cells was determined. Although, as expected, WT T cells underwent less death than $p50^{-/-}$ $cRel^{-/-}$ T cells, their survival was enhanced by Bcl-2 expression (Fig. 4 C). Of ~24% $p50^{-/-}$ $cRel^{-/-}$ T cells infected with MIG-GFP, only 8% survived after 2 d in culture (Fig. 4 C). In contrast, survival of Bcl-2 retrovirus-infected $p50^{-/-}$ $cRel^{-/-}$ T cells was dramatically enhanced compared with MIG-infected T cells. Together with the above findings, these results suggest that increased susceptibility of $p50^{-/-}$ $cRel^{-/-}$ cells to apoptosis is due to impaired TCR-induced expression of Bcl-2 family members.

IL-2-induced Survival Pathway Does Not Require p50+cRel. T cell proliferation and survival is dependent on TCR+CD28– and cytokine- (e.g., IL-2) driven pathways. During early stages of activation, before significant generation of antiapoptotic cytokines and/or expression of cytokine receptors, TCR-driven pathways predominate.

As described above, increased susceptibility of $p50^{-/-}$ $cRel^{-/-}$ cells to apoptosis is likely due to impaired TCR-induced expression of Bcl-2 family members. In addition, impaired IL-2 expression may also contribute to increased susceptibility of $p50^{-/-}$ $cRel^{-/-}$ T cells to apoptosis (discussed later). Although the addition of IL-2 did not prevent $p50^{-/-}$ $cRel^{-/-}$ or WT T cell death during initial stages of TCR-induced activation (Fig. 2 A), this could be due to lack of sufficient IL-2Rα expression. Here we have investigated a possible biochemical requirement for NF-κB in the IL-2–induced survival pathway in T cells activated with αCD3+αCD28 for 3 d. As shown above, expression of IL-2Rα is significantly enhanced in both WT and $p50^{-/-}$ $cRel^{-/-}$ T cells activated with αCD3+αCD28 for 3 d (Fig. 3 C and see below). These activated T cells were further cultured in the presence or absence of IL-2 for 1 or 2 d. As shown in Fig. 5, survival of both activated WT cells and $p50^{-/-}$ $cRel^{-/-}$ was dramatically enhanced by IL-2. Thus, although IL-2Rα expression is a little lower in $p50^{-/-}$ $cRel^{-/-}$ T cells (Fig. 3 C), it is sufficient for potent enhancement of survival by IL-2. However, this does not preclude that other aspects of IL-2 function are also unaffected in $p50^{-/-}$ $cRel^{-/-}$ T cells. Consistent with the above results, we have found that Bcl-2 family members, previously shown to be induced by IL-2 (16, 51), were also induced by IL-2 in $p50^{-/-}$ $cRel^{-/-}$ T cells (unpublished data). Therefore, these results indicate an essential requirement for NF-κB in the TCR-induced but not IL-2–induced survival pathway.

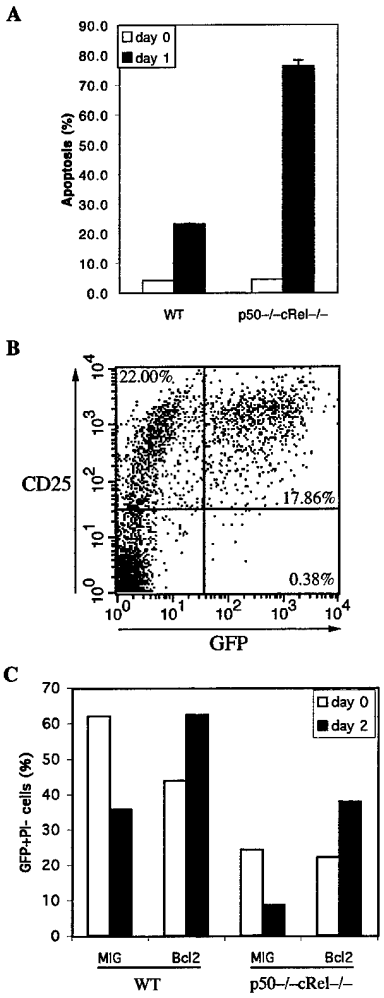

Figure 4. High susceptibility of activated $p50^{-/-}$ $cRel^{-/-}$ T cells to cell death can be rescued by Bcl-2. (A) WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells were activated by plate-coated αCD3+αCD28 for 3 d after which dead cells were removed on a Ficoll gradient. These cells were either stained with PE-αCD25 and SYTOX immediately (day 0) or cultured in T cell medium without αCD3 or IL-2 for 24 h before PE-αCD25 and SYTOX staining (day 1). Apoptosis rate represent the percentage of $CD25^+$ $SYTOX^+$ cells (apoptotic) in the total $CD25^+$ population. (B) $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells were infected with MIG retrovirus during a 4-d stimulation in the presence of αCD3+ αCD28. On day 4, viable cells were stained by αCD25 before FACS® analysis. (C) MIG and Bcl2 retroviral-infected WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells were obtained as described in B, and either stained with PI and analyzed by FACS® immediately (day 0) or cultured in T cell medium without αCD3 or IL-2 for 2 d before PI staining and FACS® (day 2). The viable infected cells were GFP^+ PI^-. The percentage was calculated based on the percentage of GFP^+ PI^- cells in the total cell population. The increase in the percentage of Bcl-2–infected cells after 2 d in this experiment is likely due to the decrease in the number of uninfected cells because of cell death, rather than an increase in the absolute number of Bcl-2–infected cells.

Constitutive NF-κB Activation Is Sufficient to Promote Activated T Cell Survival. Our results indicate that NF-κB activation by TCR engagement is necessary for T cell survival. Next, we determined whether NF-κB is sufficient to promote activated T cell survival in the absence of stimulation. We did this by using a complementation approach involving retroviral transduction of WT T cells with a constitutively active mutant (EM, refer to Materials and Methods) of the NF-κB–activating IκB kinase β (CA-IKKβ; reference 39). Nuclear NF-κB activity was readily detected in WT $CD4^+$ T cells immediately after activation. However, after a 12-h incubation without αCD3, αCD28, nor IL-2, NF-κB complex 1 completely disappeared

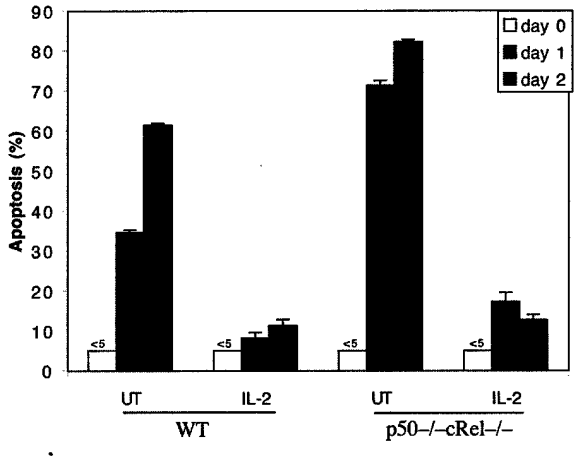

Figure 5. IL-2–induced survival pathway does not require p50+cRel. αCD3+αCD28–activated WT and $p50^{-/-}$ $cRel^{-/-}$ $CD4^+$ T cells were cultured in T cell medium alone or in the presence of 20 ng/ml IL-2 for 1 or 2 d. Cell death was determined by DNA content staining and sub-G_0 quantification.

whereas complex 2 was also reduced (Fig. 6 A). As shown in Fig. 6 B, MIG/GFP-infected T cells also showed almost complete loss of NF-κB activity after 12 h (infection efficiencies in this experiment and others described later varied from 30 to 40%). In contrast, CA-IKKβ–expressing T cells were able to maintain high NF-κB levels after 12 h.

We then tested the effect of continued presence of NF-κB on T cell survival. As shown above, cessation of TCR engagement leads to the induction of activated T cell death. After 3 d without stimulation, the number of MIG-infected T cells was greatly reduced (Fig. 6, C and D). In striking contrast, IKKβ-infected T cells showed no loss of viability (Fig. 6, C and D). As expected, retrovirus-mediated expression of Bcl-2 also substantially enhanced survival compared with MIG-infected T cells (Fig. 6, C and D). Significantly, only survival of IKKβ-infected but not uninfected T cells was enhanced (Fig. 6 C). These results indicate that the survival-promoting effect of IKKβ is likely mediated by a cell-intrinsic mechanism rather than through enhancement of cytokine production (e.g., IL-2), which would also be expected to affect survival of uninfected cells (also see below). These results suggest that the maintenance of NF-κB activity is sufficient for promoting T cell survival, likely by a cell-intrinsic mechanism.

Next, we determined whether enhanced survival of IKKβ-expressing T cells was due to an increase in the expression of Bcl-2 family members. Both Bcl-2 and Bcl-xL mRNAs were readily detected in T cells immediately after activation (Fig. 6 E). However, a 12-h incubation of MIG-infected cells led to a significant reduction in expression of both Bcl-2 and Bcl-xL. In contrast, IKKβ expression was sufficient to maintain expression of both genes (Fig. 6 E). Thus, NF-κB activity is sufficient for maintaining expression of these key antiapoptotic genes. Interestingly, although Bcl-2 and Bcl-xL mRNAs levels were greatly reduced by 12 h, significant reduction in Bcl-2 and Bcl-xL protein levels were only seen after 2–3 d (unpublished data). Thus, the loss of Bcl-2 and Bcl-xL protein levels better correlate with the kinetics of T cell death (Fig. 6, C and D). We also tested whether activated T cell death induced after antigen deprivation could be inhibited by IKKβ. To this end, OVA-specific OT-II T cells (36) were infected with MIG or IKKβ during a 3-d stimulation with antigen. Significantly, cell death induced after antigen deprivation was also inhibited by IKKβ (Fig. 6 F). Because IL-2 is an NF-κB target gene, we used OT-II T cells to determine whether IL-2 was required for IKKβ-induced enhancement of survival. As shown in Fig. 6 F, survival of

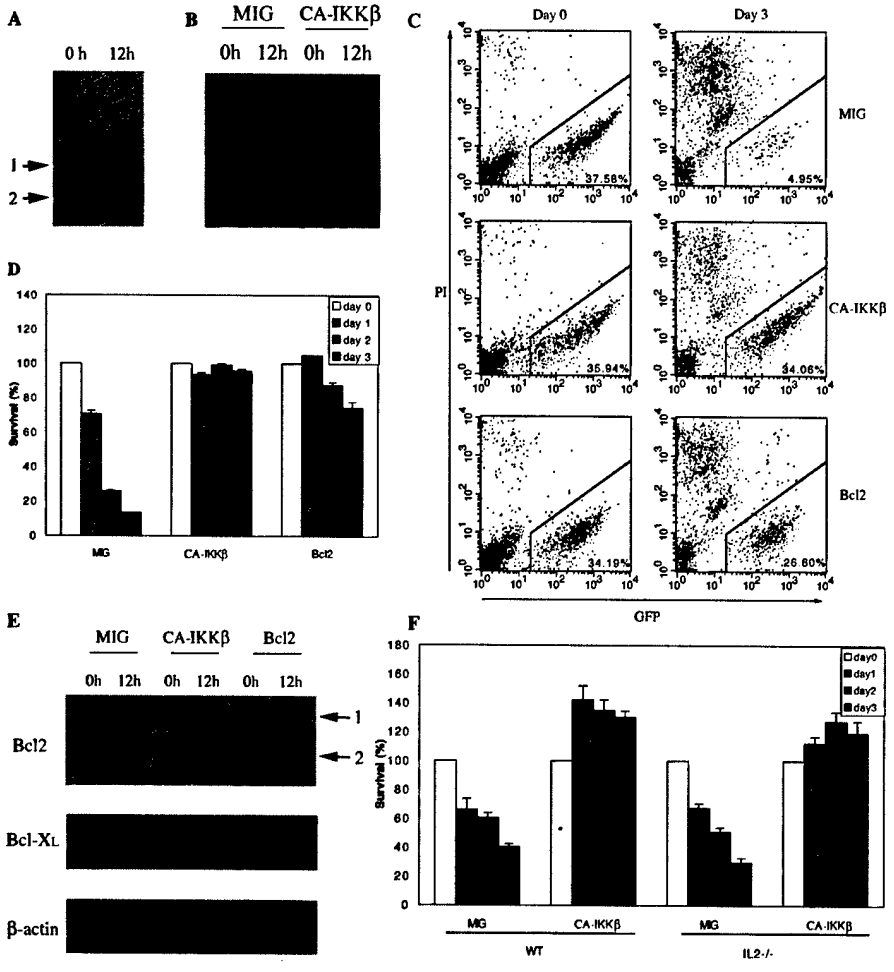

Figure 6. NF-κB activation is sufficient to promote T cell survival. (A) αCD3+αCD28+IL-2–activated WT $CD4^+$ T cells were either used to make nuclear extract immediately or cultured in T cell medium without stimulation for 12 h before nuclear extract was made. EMSA was performed with an H2 site probe. The two complexes are described in the text. (B) MIG and CA-IKKβ retrovirus-infected WT $CD4^+$ T cells were used immediately (0 h) or cultured in T cell medium without stimulation for 12 h. Nuclear extracts were made and EMSA was performed with the H2 site probe. (C and D) MIG, CA-IKKβ, and Bcl2 retrovirus-infected WT T cells were cultured in T cell medium without stimulation for 0, 1, 2, or 3 d, after which cells were stained with PI and analyzed by FACS®. FACS® data on days 0 and 3 are shown in C. The percentage indicates the proportion of GFP^+ PI^- cells in total cell population. The percentage survival of infected T cells from days 0 to 3 are shown in D. (E) MIG, CA-IKKβ, and Bcl2 retroviral-infected WT T cells were cultured in T cell medium without αCD3 or IL-2 for 12 h. RNA was extracted before and after culturing. Bcl2, Bcl-XL, and β actin expression was examined by Northern blotting. 1, endogenously expressed Bcl2; 2, ectopically expressed Bcl2. (F) MIG, CA-IKKβ retrovirus-infected OT-II×IL-$2^{+/+}$ and OT-II×IL-$2^{-/-}$ T cells were cultured in T cell medium without stimulation for 0–3 d. Survival rate of the infected cells was determined by PI staining and FACS® analysis as in C and D.

OT-II IL-2$^{-/-}$ could also be enhanced by IKKβ. Together, these results lead us to two key conclusions: (a) NF-κB activation is sufficient to promote T cell survival by an IL-2–independent and likely cell-intrinsic mechanism, and (b) down-regulation of NF-κB after termination of TCR engagement may play a crucial role in inducing activated T cell death.

Lack of Involvement of p50+cRel in Akt-induced T Cell Survival. Similar to other cell types, Akt has been shown to play an important role in regulating the survival of T cells. NF-κB is thought to be one of the key mediators of Akt-induced inhibition of cell death (18, 21). Furthermore, Akt-induced NF-κB activation has been shown to require IKKβ (20). Using p50$^{-/-}$ cRel$^{-/-}$ T cells, we also investigated a possible role of NF-κB in Akt-mediated control of T cell survival. Infection of WT T cells with constitutively active (myr mutant) Akt (15) or CA-IKKβ–encoding retrovirus substantially inhibited cell death (Fig. 7). On the other hand, IKKβ expression in p50$^{-/-}$ cRel$^{-/-}$ T cells did not inhibit cell death (Fig. 7), further demonstrating the absence of functional NF-κB activity in these cells. In striking contrast, Akt expression significantly inhibited cell death of p50$^{-/-}$ cRel$^{-/-}$ cells (Fig. 7). Therefore, these surprising results suggest a lack of requirement for NF-κB in regulating the Akt-induced survival pathway in T cells (see Discussion). Thus, NF-κB and Akt-induced pathways may independently regulate survival of T cells.

Impaired Antigen Responsiveness In Vivo and Decreased Proportion of Effector/Memory and Regulatory T Cells in p50$^{-/-}$ cRel$^{-/-}$ Mice. Very little is known about the function of NF-κB proteins in regulating T cell responses in vivo. Although the results described above indicate an important role for NF-κB in regulating T cell function in vitro, in vivo regulatory mechanisms can often be different. To test the in vivo role of NF-κB in T cell responses, mice were challenged with the superantigen SEB, a widely used reagent to study antigen-induced responses in vivo (52, 53). SEB specifically binds to TCRs that contain the Vβ8 element and drive their expansion (52). SEB-mediated T cell responses require TCR expression on T cells and MHC II expression on APC, notably DCs. Significantly, p50$^{-/-}$ cRel$^{-/-}$ mice have normal DC development and MHC II expression on spleen DCs (unpublished data and reference 27). Both WT and p50$^{-/-}$ cRel$^{-/-}$ mice were injected intravenously with SEB and the percentage of CD4$^+$ Vβ8$^+$ T cells in total CD4$^+$ T cells was determined at days 0 (uninjected), 3, and 6. In addition, CD4$^+$ Vβ6$^+$ T cell numbers were also determined at the same periods as negative controls. SEB does not bind to TCRs containing this Vβ element. As shown in Fig. 8 A, in WT mice, the CD4$^+$ Vβ8$^+$ T cell population increased from ~24 to ~32% 3 d after SEB injection and then dropped to ~22% on day 6. The kinetics of these changes in cell numbers is consistent with previous reports (53). The initial increase in Vβ8$^+$ T cells results from a proliferative response (day 3) whereas the decrease is due to cell death (day 6; reference 53). The CD4$^+$ Vβ6$^+$ T cell population remained unchanged during the same period, demonstrating the specificity of the SEB response for Vβ8$^+$ T cells. In contrast to their WT counterparts, CD4$^+$ Vβ8$^+$ T cell populations in p50$^{-/-}$ cRel$^{-/-}$ mice showed no increase after SEB injection. Instead, over a 6-d period, Vβ8$^+$ T cells dropped from ~23 to ~16% (Fig. 8 A). The CD4$^+$ Vβ6$^+$ T cells in these mice were not altered during the same period. These results support our in vitro findings and suggest that impaired SEB responsiveness of p50$^{-/-}$ cRel$^{-/-}$ T cells in vivo is due to defects in proliferation and survival.

It is generally believed that memory T cells are derived from a small subset of effector T cells activated during a primary immune response (54, 55). The proportion of memory T cells in unchallenged mice increases with age, likely through interactions with environmental antigens. Because p50$^{-/-}$ cRel$^{-/-}$ T cells showed impaired proliferation and survival after activation in vivo, we investigated whether memory T cell development was affected in these mice. Spleen cells from 3- and 8-wk-old WT and p50$^{-/-}$ cRel$^{-/-}$ mice were stained with CD4, CD44, and CD62L antibodies and FACS® analysis was performed on gated

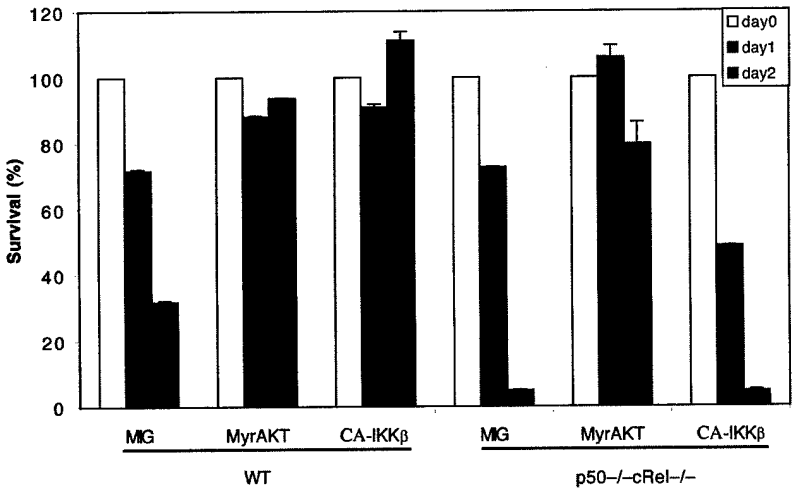

Figure 7. Lack of involvement of p50+cRel in Akt-induced T cell survival. MIG, myr-AKT, and CA-IKKβ retrovirus-infected WT and p50$^{-/-}$ cRel$^{-/-}$ CD4$^+$ T cells were cultured in T cell medium without αCD3 or IL-2 for 1 or 2 d. Survival rate of the infected cells was determined by PI staining and FACS® analysis as in Fig. 6, C and D.

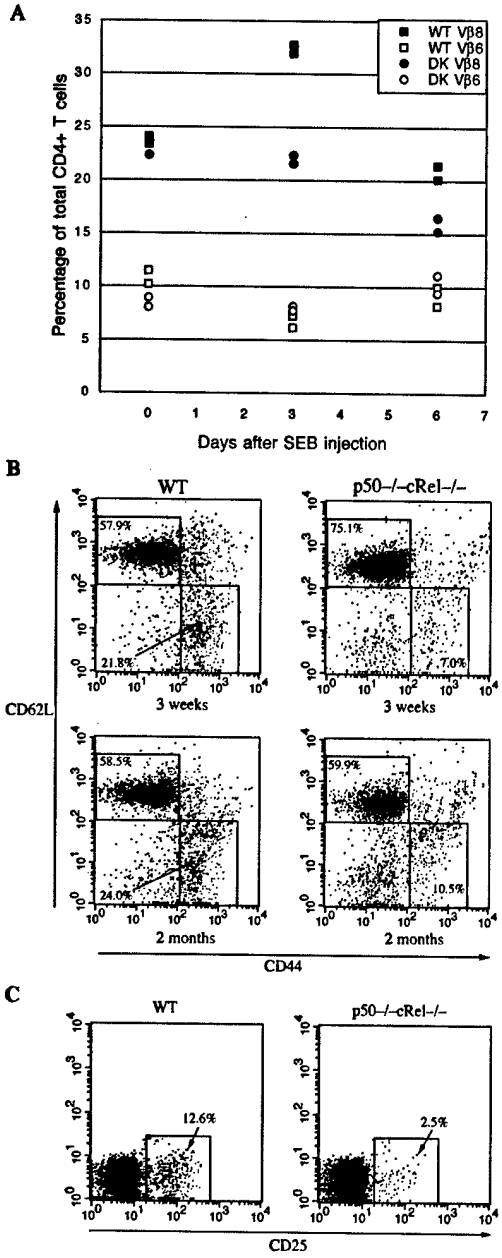

Figure 8. Impaired antigen-induced responses and effector/memory and regulatory T cell generation in WT and $p50^{-/-}$ $cRel^{-/-}$ mice. (A) WT and $p50^{-/-}$ $cRel^{-/-}$ mice were either uninjected (day 0) or injected with SEB. $V\beta8^+$ and $V\beta6^+$ T cell populations were determined 3 and 6 d after injection. Two mice of each genotype were used per condition. (B) Naive and memory T cell populations were determined using 3- and 8-wk-old WT and $p50^{-/-}$ $cRel^{-/-}$ mice. Splenocytes from these mice were stained with CD4, CD44, and CD62L antibodies. FACS® analysis was performed on gated $CD4^+$ T cells. Naive T cells were $CD44^{low}$ $CD62L^+$ and memory T cells were $CD44^{high}$ $CD62L^-$. (C) Splenocytes from 2-mo-old WT and $p50^{-/-}$ $cRel^{-/-}$ mice were stained with CD4 and CD25. FACS® analysis was performed on gated $CD4^+$ T cells.

$CD4^+$ cells. As shown in Fig. 8 B, naive T cells were identified as the $CD44^{low}$ $CD62L^+$ population and memory T cells as the $CD44^{high}$ $CD62L^-$ population (55). Strikingly, 3-wk-old WT mice had approximately threefold more effector/memory T cells than $p50^{-/-}$ $cRel^{-/-}$ mice (21.8 vs. 7%) whereas in 8-wk-old mice the difference was ~2.5-fold (24 vs. 10.5%). Thus, the proportion of memory T cells is significantly reduced in $p50^{-/-}$ $cRel^{-/-}$ mice. We also tested regulatory T cell ($CD4^+$ $CD25^+$) populations in WT and $p50^{-/-}$ $cRel^{-/-}$ mice. In 2-mo-old $p50^{-/-}$ $cRel^{-/-}$ mice, $CD4^+$ $CD25^+$ T cells were reduced fivefold compared with WT mice (Fig. 8 C). Significantly, the differentiation of naive T cells into effector, memory, and regulatory T cells requires initial antigen-induced activation. Therefore, the decreased numbers of all three populations in $p50^{-/-}$ $cRel^{-/-}$ mice are consistent with our results showing impaired in vitro and in vivo proliferation and survival of $p50^{-/-}$ $cRel^{-/-}$ T cells. Together, these results show for the first time an important role for NF-κB in regulating mature T cell function in vivo.

Discussion

Multiple signaling pathways are induced during mature T cell activation that together regulate proliferation, survival, and cytokine production. However, the precise role of specific signaling molecules and transcription factors in regulating these pathways is not well understood. To this end, we have studied the role of the NF-κB transcription factor in mature T cells by using doubly deficient $p50^{-/-}$ $cRel^{-/-}$ T cells, which exhibit virtually no TCR-inducible κB site binding activity. Our results demonstrate an essential role for NF-κB in regulating cell cycle entry and survival of activated T cells in vitro and in vivo. These results indicate that of the many transcription factors considered important in T cells, NF-κB might be among the most crucial. The findings reported here help us understand many key aspects of regulatory mechanisms involved in T cell function.

Regulation of T Cell Survival by NF-κB. A primary goal of this study was to elucidate NF-κB–dependent mechanisms involved in regulating T cell survival. We have found, using both in vitro and in vivo approaches, that NF-κB activation after TCR engagement plays a crucial role in regulating T cell survival. Significantly, our results indicate that NF-κB activation is not only necessary but also sufficient for T cell survival. We have also found that NF-κB plays an essential role in TCR-induced regulation of Bcl-2 and Bcl-xL gene expression. Significantly, the high susceptibility of activated $p50^{-/-}$ $cRel^{-/-}$ T cells to apoptosis was inhibited by retroviral expression of Bcl-2, suggesting that NF-κB prevents cell death by regulating Bcl-2 family member expression. These findings establish a critically important function of NF-κB in TCR-induced regulation of survival. Our results also demonstrate that the combined absence of p50 and cRel subunits is required for significant impairment of mature T cell function. Although $cRel^{-/-}$ T cells were thought to have greatly impaired proliferation (35), we have previously found relatively intact responsiveness in these and $p50^{-/-}$ T cells (28). Thus, our findings with $p50^{-/-}$ $cRel^{-/-}$ T

cells indicate redundant functions for these two proteins in mature T cells.

$p50^{-/-}$ $cRel^{-/-}$ T cells show impaired IL-2 expression after activation. Thus, the antiapoptotic function of NF-κB may involve both TCR induction of expression of Bcl-2 family members and IL-2. We believe both these NF-κB–dependent mechanisms are important, but at different stages. During the initial TCR-dependent phase of activation (1–2 d), i.e., before there is significant generation of antiapoptotic cytokines and/or expression of cytokine receptors, NF-κB directly induces expression of Bcl-2 family members and enhances activated T cell survival (Fig. 2). The ability of NF-κB to enhance survival in a cytokine-(e.g., IL-2) independent manner is further evidenced by IKKβ transduction studies with WT and $IL\text{-}2^{-/-}$ T cells (Fig. 6). At this early stage, T cell survival might be regulated by cooperative interactions between NF-κB and other TCR+CD28–induced pathways. During later stages of T cell activation (3–4 d), cytokine-driven responses likely predominate. At this stage, NF-κB may also enhance survival through generation of antiapoptotic cytokines, such as IL-2. Interestingly, we have shown a lack of involvement of NF-κB in the IL-2–induced survival pathway. Thus, although NF-κB is important for IL-2 expression, it is not a mediator of IL-2–induced survival, which also depends on Bcl-2 family members. Thus, T cell survival can be controlled independently or through cooperative regulation of Bcl-2 family expression by TCR+CD28 and cytokine-induced pathways. It was recently shown that T cell activation and generation of memory/effector T cells can still occur in γc-deficient mice (56), which are nonresponsive to many cytokines important for T cell function including IL-2, IL-4, IL-7, and IL-15. It is interesting to speculate whether immune responses in these mice are mediated largely by TCR-induced NF-κB activation.

A key question is why TCR engagement induces an antiapoptotic pathway in the first place. One reason might be to block simultaneously induced apoptotic pathways for T cell expansion to occur. It has recently been shown that TCR signals lead to activation of Bim, a proapoptotic member of the Bcl-2 family (7, 57). TCR signals also lead to generation of reactive oxygen species in activated T cells, which contribute to induction of apoptosis (58). This would be consistent with the function of Bcl-2 proteins, which are known to inhibit apoptosis induced by reactive oxygen species (59, 60). However, an additional function of antiapoptotic pathways may also allow efficient removal of activated T cells once TCR stimulation ends. Because continued antiapoptotic pathway activation will depend on TCR signaling, this would allow rapid elimination of T cells when TCR engagement ends. Such cell death may occur as a result of down-regulation of one or multiple antiapoptotic signaling pathways. We have shown here that nuclear NF-κB levels rapidly decline in WT T cells after cessation of TCR stimulation, an event that was concomitant with induction of cell death. However, T cells complemented with a retrovirus-encoding CA-IKKβ maintained nuclear NF-κB levels and survived in the absence of stimulation. Thus, NF-κB activation is not only sufficient to promote T cell survival but down-regulation of NF-κB can also provide a mechanism for induction of cell death. Although IKKβ expression apparently enhances survival in a cell-intrinsic manner by inducing Bcl-2 family member expression, it is likely that down-regulation of NF-κB after termination of TCR engagement leads to apoptosis both through decreased expression of Bcl-2 proteins and cytokines such as IL-2.

The PKB/Akt kinase is a key mediator of T cell survival pathways and has previously been shown to function through NF-κB (19–21, 61). However, our results show that Akt but not IKKβ is fully capable of enhancing $p50^{-/-}$ $cRel^{-/-}$ T cell survival. In addition, we have found that the PI3K inhibitor LY294002, which inhibits Akt activation, does not inhibit αCD3+αCD28–induced NF-κB activation in WT T cells (unpublished data). Furthermore, IL-2 induces Akt but not NF-κB activation in T cells (unpublished data). Together, these different findings strongly suggest that NF-κB may not be a mediator of the Akt-induced survival pathway. Therefore, these results indicate the existence of two parallel and potentially independent survival pathways in T cells. They may also help explain how IL-2, which activates Akt, enhances the survival of $p50^{-/-}$ $cRel^{-/-}$ T cells. In addition, Akt is also thought to be a key mediator of CD28-induced responses. However, CD28-induced responses are generally evident only in the presence of TCR engagement. Because TCR-induced NF-κB itself is so crucial for survival, it is difficult to determine the specific role of NF-κB in the CD28 survival pathway using $p50^{-/-}$ $cRel^{-/-}$ T cells (the role NF-κB in CD28-induced proliferation is discussed below). The potentially NF-κB–independent nature of Akt prosurvival function suggests the existence of alternate Akt-induced mechanisms for regulating Bcl-2 family member expression. Although the identity of such transcription factors is presently unknown, they may include members of signal transducer and activator of transcription, Ets, or AP-1 families. Interestingly, we have also found that activated $p50^{-/-}$ $cRel^{-/-}$ T cells, similar to T cells deficient in $p50^{-/-}$, $RelA^{-/-}$, or $cRel^{-/-}$ (28), are no more susceptible to Fas-induced killing than WT T cells (unpublished data). These results indicate that a distinct survival pathway might be required for regulating Fas killing and further underscore a specific function for NF-κB in regulating TCR-induced survival.

Immunological adjuvants were shown to induce expression of the IκB family member Bcl-3 in T cells, resulting in enhanced T cell survival (62). These results, together with our findings, suggest that Bcl-3 likely enhances transcriptional functions of NF-κB in T cells. One interesting possibility might be that Bcl-3 promotes T cell survival by maintaining NF-κB nuclear activity in the absence of TCR engagement. Gene targeting studies of PKCθ and Bcl-10 have revealed an essential role for these proteins in NF-κB activation and in regulating T cell proliferative responses (32, 33). Based on our results with $p50^{-/-}$ $cRel^{-/-}$ T cells, it is possible that impaired proliferation of T cells deficient

in PKCθ or Bcl-10 might be due to defects in both cell division and cell survival.

Control of T Cell Proliferation and Effector/Memory and Regulatory T Cell Generation by NF-κB. We have found that in addition to regulating survival, NF-κB proteins also regulate $CD4^+$ T cell proliferation. TCR-induced cell cycle entry of $p50^{-/-}$ $cRel^{-/-}$ T cells is both significantly reduced and delayed compared with WT T cells. Previous studies have implicated NF-κB as a key component of the CD28 costimulatory pathway (63, 64). However, our results indicate that CD28 could significantly increase proliferation of $p50^{-/-}$ $cRel^{-/-}$ T cells, suggesting that NF-κB control of proliferation might be more specific for the TCR pathway. One possibility is that CD28-induced enhancement of $p50^{-/-}$ $cRel^{-/-}$ T cell proliferation is through Akt-induced pathways. In addition to NF-κB, CD28 also induces activation of the AP-1 family of transcription factors. Thus, AP-1 factors may play a more crucial role in the CD28 pathway than NF-κB and CD28-induced AP-1 activity may synergize with TCR-induced NF-κB and NFAT pathways in regulating T cell proliferation, survival, and other functions. Interestingly, $PKC\theta^{-/-}$ T cells are deficient in both NF-κB and AP-1 activation (33). Comparative analysis of T cell function in $PKC\theta^{-/-}$ and $p50^{-/-}$ $cRel^{-/-}$ mice may therefore help us better understand the specific role played by AP-1 in regulating T cell proliferation.

IL-2 expression was significantly reduced in $p50^{-/-}$ $cRel^{-/-}$ cells, but the addition of exogenous IL-2 failed to rescue proliferation defects. $p50^{-/-}$ $cRel^{-/-}$ cells, however, express IL-2R and are protected from cell death by IL-2. Thus, impaired proliferative responses cannot simply be due to reduced IL-2 or IL-2R expression, but instead may result from impaired TCR induction of genes involved in cell cycle control. The identity of such NF-κB–regulated genes has yet to be determined. One of the important findings reported here is the decreased number of effector/memory and regulatory T cells in $p50^{-/-}$ $cRel^{-/-}$ mice. Because differentiation of naive T cells into effector, memory, and regulatory T cells requires antigen-induced activation, these results provide further evidence for the crucial role of NF-κB in regulating activation-induced proliferation and survival of T cells. However, our results also indicate that a certain proportion of effector/memory T cells are generated in $p50^{-/-}$ $cRel^{-/-}$ mice, which appear to increase with age. One possibility is that decreased effector/memory T cell in $p50^{-/-}$ $cRel^{-/-}$ mice are a consequence of impaired proliferation and/or survival during a primary response. However, the small number of effector/memory T cells that do form in $p50^{-/-}$ $cRel^{-/-}$ mice may undergo expansion by cytokine-driven homeostatic mechanisms, which might not require NF-κB.

NF-κB Function in T Cell Development. Consistent with our findings, previous studies of IκB-Tg mice have also shown impaired proliferative responses and IL-2 expression, but unlike $p50^{-/-}$ $cRel^{-/-}$ mice, IκB-Tg mice also showed impaired thymocyte development and reduction in numbers of peripheral T cells (29–31). The effect was especially pronounced for the CD8 lineage (29–31). As recently shown, impaired thymocyte development likely reflects a requirement for NF-κB in regulating survival of developing thymocytes (65). Unlike mature T cells (Fig. 1 E), $p50^{-/-}$ $cRel^{-/-}$ thymocytes still exhibit low levels of RelA (unpublished data). Thus, RelA may play an important role in the development of thymocytes in the absence of p50 and cRel. Notably, a proapoptotic role for NF-κB in thymocytes has also been proposed (30, 66). Thus, although the findings presented here demonstrate a crucial role for NF-κB in mature T cells, the precise role played by NF-κB in thymocytes remains to be determined.

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