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Targeted Disruption of the p50 Subunit of NF-κB Leads to Multifocal Defects in Immune Responses

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Summary

NF-κB, a heterodimeric transcription factor composed of p50 and p65 subunits, can be activated in many cell types and is thought to regulate a wide variety of genes involved in immune function and development. Mice lacking the p50 subunit of NF-κB show no developmental abnormalities, but exhibit multifocal defects in immune responses involving B lymphocytes and nonspecific responses to infection. B cells do not proliferate in response to bacterial lipopolysaccharide and are defective in basal and specific antibody production. Mice lacking p50 are unable effectively to clear L. monocytogenes and are more susceptible to infection with S. pneumoniae, but are more resistant to infection with murine encephalomyocarditis virus. These data support the role of NF-κB as a vital transcription factor for both specific and nonspecific immune responses, but do not indicate a developmental role for the factor.

Introduction

NF-κB is a heterodimeric transcription factor composed of p50 and p65 subunits that was originally identified as an inducible B cell–specific factor able to bind to the κB motif in the intronic κ light chain enhancer (Sen and Baltimore, 1986a, 1986b). On the basis of the correlation between induction of NF-κB and κ light chain gene expression, NF-κB was thought to be a key regulatory molecule in κ light chain gene activation and perhaps to be involved in the maturation of pre–B to B cells (Sen and Baltimore, 1987). NF-κB is now recognized to be a ubiquitously expressed factor that is present in most cell types in an inducible cytoplasmic form (for reviews see Grilli et al., 1991; Grimm and Baeuerle, 1993). A wide range of stimuli, including viruses, bacterial lipopolysaccharide (LPS), engagement of antigen receptors, stress factors, and cytokines lead to translocation of NF-κB from the cytoplasm to the nucleus, where it appears in active form capable of binding decameric κB sequence motifs of the general form 5′-GGGRNNYYCC-3′. An extensive set of genes with putative NF-κB-binding sites has been identified, and in many of these, the NF-κB sites appear crucial to the regulation of transcription. Putative cellular target genes are largely involved in the acute-phase response, inflammation, lymphocyte activation, and cell growth and differentiation. These genes include cell-surface molecules involved in immune function such as immunoglobulin κ light chain, class I and II major histocompatibility complex (MHC), and cytokines such as interleukin-1β (IL-1β), IL-2, IL-6, interferon-β (IFNβ), and tumor necrosis factor α (TNFα).

Following cloning of genes encoding the p50 and p65 subunits of NF-κB (Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991), it became evident that p50 and p65 were members of a larger NF-κB/Rel family of transcription factors. There are currently five members of the NF-κB/Rel family that have been identified in vertebrates: NFKB1 (p50 and its precursor, p105), NFKB2 (p52 and its precursor, p100), p65 (RelA), c-Rel (Rel), and RelB (for review see Nolan and Baltimore, 1992). Most of these family members can heterodimerize in vitro. As dimers, all five proteins can form complexes with κB DNA sequence motifs, and all have been shown to affect transcription of κB reporter genes positively or negatively when assayed following transfection.

Despite extensive knowledge of the biochemical and molecular properties of these family members, little is known about the roles of these transcription factors in vivo (for review see Liou and Baltimore, 1993). The control of different heterodimers within cells, their tissue- and cell type–specific distribution and regulation within developing organisms, and the relationship of different heterodimeric complexes to the in vivo regulation of transcription of individual genes and regulatory gene networks are all poorly understood issues. These issues all relate to a central unresolved problem of how so many diverse input signals are coherently coupled to so many different transcriptional responses through the NF-κB/Rel family.

To develop an understanding of the physiological roles of NF-κB, we have initiated a gene targeting program to generate mice lacking individual and multiple subunits. Here we report the effects of ablating the portion of the *NFKB1* gene encoding the p50 subunit. The in vivo consequences of this gene targeting on both gene expression and induction of NF-κB reveal complex focal defects in NF-κB regulation that were not apparent in previous in vitro studies. Surprisingly, many genes with apparent binding sites for NF-κB and even for p50 homodimer are expressed normally by cells lacking p50. Our results indicate that the p50 subunit of NF-κB does not play a development role in the immune system (or elsewhere), but is a critical component of gene programs regulating both responses of mature lymphocytes and nonspecific immune responses to pathogens.

Results

Targeting the *NFKB1* Gene and Generation of Mutant p50$^{-/-}$ Mice

The p50 subunit of NF-κB is not synthesized as an active DNA-binding protein, but is generated by proteolytic processing of a 105 kDa precursor called p105 that contains p50 in its N-terminal half (Ghosh et al., 1990; Kieran et al., 1990) and IκB-γ in its C-terminal half (Inoue et al., 1992; Liou et al., 1992). The *NFKB1* gene encodes both p105 and IκB-γ mRNAs, probably through separate promoters. Members of the NF-κB/Rel family of transcription factors share a conserved N-terminal 300 amino acid Rel homology domain responsible for both dimerization and binding to κB sequence motifs (Grimm and Baeuerle, 1993; Logeat et al., 1991). The construct pp50KO used for targeted disruption of the *NFKB1* gene was designed to insert a PGK–*neo* cassette into exon 6 of the *NFKB1* gene (Figure 1A). Exon 6 encodes residues 134–187 that lie within the Rel homology domain that extends from residues 30–330. This disruption would be expected to produce a truncated polypeptide that had no ability to bind DNA or dimerize with itself or other NF-κB/Rel family members. Targeted *NFKB1* alleles should be unable to direct synthesis of the p105 precursor to p50, but should not affect IκB-γ synthesis from an internally promoted mRNA.

The linearized targeting vector was electroporated into E14 embryonic stem cells, and the average frequency of homologous recombination was about 1 in 25 G418^{R} colonies. Several clones were used to generate chimeric mice, and homozygous mice were generated by intercrosses of heterozygous mice (Figure 1B). To verify that the gene disruption created a null mutation, we examined expression of the p105 protein in spleen (Figure 1C). In tissue lysates from mice that were genotyped as homozygous for the disrupted *NFKB1* allele, both the p105 precursor and processed p50 protein were undetectable by an anti-p50 serum. These results indicate that p105 and p50 have been functionally inactivated in homozygous mice, which we will refer to as p50$^{-/-}$ mice.

When housed under specific pathogen-free conditions, p50$^{-/-}$ mice showed no differences in size, behavior, life span up to 1 year, and reproductive ability when compared with littermate controls. They were, however, more prone to infection and died more frequently at an earlier age when housed under conventional conditions. Both gross examination and histological analyses of all organ systems were unremarkable. In particular, lymphoid organs, including bone marrow, thymus, spleen, and lymph nodes, had normal architecture without evidence of specific pathologic changes (data not shown).

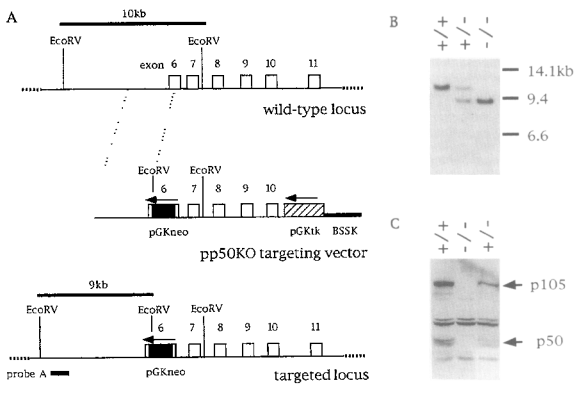

Figure 1. Production of Mice Lacking the p50 Subunit of NF-κB

(A) The *NFKB1* locus and targeting construct. Map of the region of the *NFKB1* gene encoding the Rel homology domain of p105. Exons are represented by open boxes. The length of diagnostic EcoRV restriction fragments and the probe used for Southern blot analysis are shown.

(B) Southern blot analysis of EcoRV-digested tail DNAs hybridized with probe A.

(C) Western blot analysis of spleen lysates from mice genotyped as wild type, homozygous, and heterozygous for the disrupted *NFKB1* allele. The protein blot was incubated with serum specific to mouse p50. Locations of specific p105 and p50 bands are indicated by arrows.

Normal Mature B and T Cell Subsets

The normal size and follicular architecture of lymph nodes and spleens from p50$^{-/-}$ mice suggested that lymphocyte populations would be normal. This was confirmed by flow cytometric analyses of splenocyte populations (Figure 2).

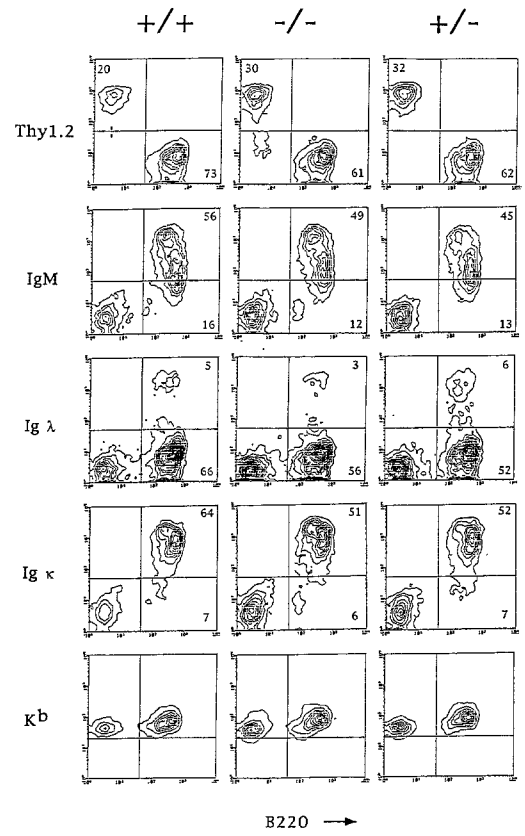

Figure 2. Normal κ Light Chain Usage, Class I MHC Expression, and Mature B and T Cell Subsets

Equivalent numbers of lymphocytes were obtained from single cell suspensions prepared from spleens of p50$^{-/-}$ and control littermates (data not shown). Splenocytes were stained with labeled antibodies and analyzed by flow cytometry as described in Experimental Procedures. Percentages of cells in relevant quadrants are indicated.

Both numbers and ratios of B and T cells isolated from spleens were normal. Analyses of B220 and Thy1.2 surface expression on splenocytes were unremarkable. When B cells were examined for surface immunoglobulin expression, normal levels of surface immunoglobulin M (IgM) and immunoglobulin D (IgD) were expressed. Interestingly, light chain usage was unchanged in $p50^{-/-}$ mice. κ light chain was still used in marked preference to λ light chain. This lack of increase in λ light chain usage indicates that the absence of p50-containing NF-κB/Rel complexes did not significantly perturb the developmental program of κ light chain expression in $p50^{-/-}$ B cells. Lymphocyte populations in bone marrow showed normal surface expression and distribution of B220 and CD43 molecules (data not shown). Splenic T cells and thymocytes from $p50^{-/-}$ mice had normal surface expression and distribution of CD3ε, CD4, CD8, and CD28 molecules (data not shown). Interestingly, the surface expression of class I and II MHC molecules on $p50^{-/-}$ lymphocytes was also normal. Expression of the K^b class I MHC molecule has been thought to involve binding of a p50 homodimer to a κB site in the K^b promoter (Baldwin and Sharp, 1988; Israël et al., 1989; Kieran et al., 1990). In addition, NF-κB-binding motifs have been identified in the genes encoding β2-microglobulin (Israël et al., 1989) and invariant chain (Pessara and Koch, 1990), proteins that are involved in class I and II MHC expression.

Composition and Induction of NF-κB/Rel Complexes in B Cells

We have previously described an ordered pattern of NF-κB/Rel complexes during different stages of B cell development: mainly p50 and p65 in pre–B (and non–B) cells, a predominance of Rel and p50 in mature B cells, and expression of p52 and RelB in plasmacytoma lines (Liou et al., 1994). We hypothesized that this sequential induction of NF-κB/Rel family proteins reflected the requirement for expression of different genes during terminal B cell differentiation. It was, therefore, of interest to define what effect the absence of p50 had on the cytoplasmic levels of other NF-κB/Rel family members in splenic B cells and to determine whether any compensation for the loss of p50 was occurring. Using antisera whose specificity have previously been established (Liou et al., 1994), we subjected cytoplasmic extracts from resting splenic B cells to Western blot analyses to determine the relative amounts of different family members that were available in the cytoplasm for induction. Cytoplasmic extracts contained equivalent amounts of p52, but amounts of Rel, RelB, and p65 were substantially lower in $p50^{-/-}$ B cells (Figure 3A). Thus, there was no evidence for up-regulation in $p50^{-/-}$ B cells of other family members, particularly p52, the closest homolog of p50. These results imply that compensation by other family members was not occurring at a substantial level in B cells. Interestingly, similar Western blot analyses of cytoplasmic extracts from control and $p50^{-/-}$ embryonic fibroblast lines revealed that levels of p52, Rel, RelB, and p65 were unchanged in $p50^{-/-}$ fibroblast lines, implying that the observed decrease in Rel, RelB, and p65 in $p50^{-/-}$ B cells was cell type specific (data not shown).

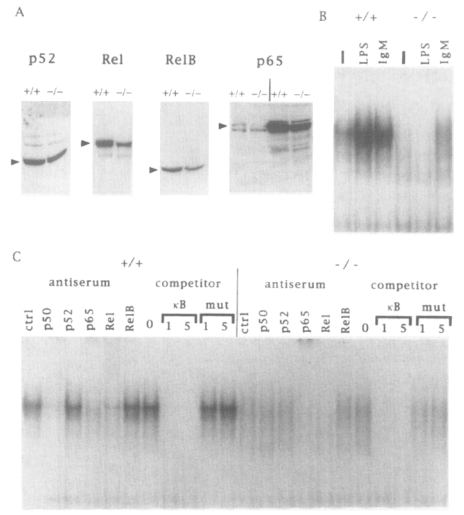

Figure 3. Composition and Induction of NF-κB/Rel Complexes in B Cells

(A) Western blot analysis of cytoplasmic extracts prepared from purified control and $p50^{-/-}$ B cells. Each protein blot was incubated with serum specific to p52, Rel, RelB, and p65. An additional preparation of control and $p50^{-/-}$ B cell cytoplasmic extracts containing less protein was included in the first two lanes of the p65 Western blot to demonstrate more clearly the difference in p65 protein levels. Arrows indicate location of specific protein bands, blockable by addition of antigen.

(B) Induction of NF-κB/Rel complexes by LPS and anti-IgM treatment. EMSA of 5 μg of nuclear extracts from small resting control and $p50^{-/-}$ B cells prepared in parallel after 4 hr in medium alone (solid line) or treatment with LPS (10 μg/ml) or anti-IgM antibody (30 μg/ml). A loop oligonucleotide probe containing the class I MHC κB site was used.

(C) Composition of NF-κB/Rel complexes induced by anti-IgM treatment in control and $p50^{-/-}$ B cells assessed by EMSA with antibody inhibition. Purified specific antibodies (5 μg) to each NF-κB/Rel component were preincubated with nuclear extracts prior to addition of the class I MHC κB oligonucleotide probe. Control (ctrl) represents normal rabbit serum. Specific complexes were inhibited by competition with 1 ng and 5 ng of unlabeled oligonucleotide containing a class I MHC κB site, but not a mutant (mut) κB site.

We were interested in testing whether the reduction in cytoplasmic NF-κB/Rel complexes in $p50^{-/-}$ B cells affected the ability of these complexes to be induced. Both bacterial LPS and engagement of antigen receptor are potent inducers of translocation of NF-κB/Rel complexes in B cells (Sen and Baltimore, 1986a; Atchison and Perry, 1987; Liu et al., 1991). To mimic antigen receptor engagement, we used anti-IgM antibody. We chose concentrations of inducers that caused equivalent induction of NF-κB/Rel complexes at 4 hr in wild-type small resting B cells that were rigorously purified from splenocytes (Figure 3B). Induction of NF-κB/Rel complexes was assayed by electrophoretic mobility shift analysis (EMSA) using equivalent amounts of nuclear extracts from treated and untreated B cells. When analyzed in parallel with control B cells, $p50^{-/-}$ B cells showed less induction of NF-κB/Rel complexes with anti-IgM treatment and no induction with LPS treatment. Interestingly, the low amount of constitutive nuclear NF-κB in wild-type cells was not evident in untreated $p50^{-/-}$ B cells.

The composition of NF-κB/Rel complexes induced by

anti-IgM antibody was determined by EMSA in which nuclear extracts were preincubated with specific antisera to individual NF-κB/Rel family members (Figure 3C). In wild-type B cells, anti-p50 serum inhibited nearly all of the binding of induced complexes, whereas anti-p65 and anti-Rel sera each only partially inhibited binding, and anti-p52 and anti-RelB sera had little effect. These results imply that p50–p65 and p50–Rel were the major NF-κB/Rel complexes induced in wild-type B cells by anti-IgM antibody. The same complexes were also induced in wild-type B cells by LPS treatment (data not shown). In contrast, the complexes induced by anti-IgM antibody in $p50^{-/-}$ B cells were composed primarily of Rel and p65 with, of course, no p50 and no evident p52. Thus, despite the similar induction of NF-κB complexes by anti-IgM and LPS in wild-type B cells, the absence of p50 revealed a striking difference in the ability of these two inducers to activate translocation of complexes containing p65 and Rel.

Induction of NF-κB/Rel Complexes Activates B Cell Proliferation

The differential ability of anti-IgM and LPS treatment to cause translocation of NF-κB/Rel complexes in $p50^{-/-}$ B cells allowed us to ask whether induction of NF-κB/Rel complexes is an important signal for B cell proliferation, since both anti-IgM antibody and LPS are potent B cell mitogens (Figure 4). In multiple experiments, no reproducible differences were observed in the proliferative responses of $p50^{+/-}$ and $p50^{+/+}$ B cells to either mitogen. Purified $p50^{-/-}$ B cells also proliferated in response to increasing amounts of anti-IgM antibody, although somewhat more weakly than control B cells. In contrast, purified $p50^{-/-}$ B cells were unresponsive to a range of LPS concentrations that stimulated vigorous proliferation of control

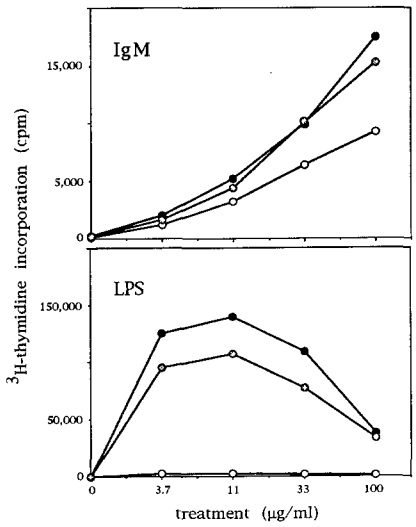

Figure 4. Induction of B Cell Proliferation Correlates with Induction of NF-κB/Rel Complexes

Proliferation of small resting B cells treated with either anti-IgM antibody or LPS. Small resting B cells were purified from spleens of $p50^{-/-}$ (open circles), $p50^{+/-}$ (stippled circles), or wild-type (closed circles) mice.

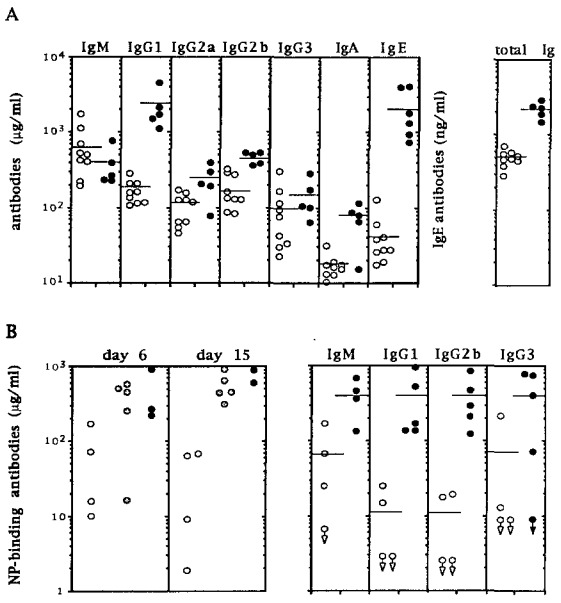

Figure 5. Resting and Specific Antibody Production

Each symbol represents the value obtained from one animal by ELISA.
(A) Serum concentrations of immunoglobulin isotypes from unimmunized control (closed circles) and $p50^{-/-}$ (open circles) mice. Total resting serum immunoglobulin levels are given in relative units.
(B) T cell–dependent antibody response. At 6 and 15 days after immunization with NP_{15}-CG, concentrations of total NP-specific antibodies in sera were determined from control wild-type (closed circles) and $p50^{+/-}$ (stippled circles) and from $p50^{-/-}$ (open circles) mice. Relative amounts of NP-specific IgM antibodies at day 6 and NP-specific antibodies of the indicated isotypes at day 15 were determined for control (closed circles) and $p50^{-/-}$ (open circles) mice.

B cells. LPS treatment of $p50^{-/-}$ B cells did, however, induce class II MHC up-regulation (data not shown), suggesting that other signal transduction pathways were still being activated by LPS in $p50^{-/-}$ B cells and that these B cells were fully capable of binding LPS. Thus, the ability of these two inducers to activate NF-κB/Rel complexes correlated with their ability to stimulate B cell proliferation. These results indicate that the induction of NF-κB/Rel complexes plays a critical role in activating transcription of genes involved in B cell mitogenic responses.

Basal and Specific Antibody Production

To assess basal production of antibodies in unimmunized mice, we measured resting serum immunoglobulin levels in $p50^{-/-}$ and control littermates. Interestingly, total serum immunoglobulin was 4-fold lower in $p50^{-/-}$ mice (Figure 5A). This decrease resulted from a significant reduction in all isotypes measured except for IgM, which was slightly elevated in $p50^{-/-}$ mice. The largest reductions were in serum IgE, which was reduced more than 40-fold; serum IgG1, which was decreased more than 10-fold; and serum IgA, which was decreased 5-fold. These results indicate that $p50^{-/-}$ mice were inefficient in the production of immunoglobulin isotypes that result from heavy chain class switching, suggesting that one key cellular event dependent on p50 is isotype switching.

The defects in resting serum immunoglobulin levels prompted us to examine whether $p50^{-/-}$ mice were able to mount effective humoral responses when immunized with specific antigens. Littermates were immunized with a T cell–dependent antigen, NP_{15}-CG, and anti-NP antibody production was measured (Figure 5B). Total NP-binding antibodies were greatly lower in $p50^{-/-}$ mice compared with control littermates both 6 and 15 days after immunization. Unlike resting serum immunoglobulin levels, in which we observed considerable variation in the reduction of individual isotypes, NP-specific antibodies of different IgG isotypes were all substantially reduced relative to control responses. Thus, the p50 subunit of NF-κB is apparently an essential component of the gene regulatory programs that direct antibody production in response to specific immunogens.

Responses to Pathogens

Given the presence of κB sites in multiple genes involved in immune and acute-phase responses, we were interested in testing whether NF-κB plays a critical role in responses to infection with different pathogens. The course of disease with major classes of infectious pathogens was examined in $p50^{-/-}$ and control mice. Responses to the pathogenic intracellular bacterium Listeria monocytogenes, the extracellular gram-positive bacterium Streptococcus pneumoniae, the extracellular gram-negative bacteria Haemophilus influenzae and Escherichia coli K1, and the murine encephalomyocarditis (EMC) virus revealed striking differences in outcome between $p50^{-/-}$ and control mice that highlight the complex involvement of p50 in transcriptional responses to infection.

L. monocytogenes, an intracellular bacterium, can survive in nonactivated macrophages. Clearance of infection requires activation of macrophages by cytokines produced by T and NK cells (Bancroft et al., 1991). When infected with L. monocytogenes, $p50^{-/-}$ mice eradicated extracellular bacteria, but were impaired in elimination of intracellular bacteria. At 6 days after intraperitoneal injection of 5000 bacteria, control mice had few or no splenic or peritoneal bacteria while $p50^{-/-}$ littermates had several thousand splenic, but no peritoneal, bacteria (Figure 6A). This defective clearance likely reflects a defect in macrophage activation, rather than a defect in phagocytic cell number or basal phagocytic activity. Both resting peritoneal macrophages and blood neutrophils from $p50^{-/-}$ mice were present in normal numbers and had normal phagocytic activity when assayed for their ability to ingest opsonized zymosan particles (data not shown). These results suggest that extracellular L. monocytogenes were phagocytosed normally in $p50^{-/-}$ mice and that the defective clearance of intracellular L. monocytogenes resulted from a defect in the activation of macrophages by cytokines.

Although $p50^{-/-}$ mice eliminated extracellular L. monocytogenes, control of the replication of an aggressive, extracellular, and gram-positive pathogen, S. pneumoniae, was compromised. In multiple experiments, when littermates were injected intraperitoneally with 100 S. pneumoniae bacteria, $p50^{-/-}$ mice typically died around 24 hr, while control littermates took between 36 and 72 hr to succumb, with occasional control animals surviving (Figure 6B). A small increase in survival time for wild-type over $p50^{+/-}$ littermates was the only difference we consistently observed in any response between $p50^{+/-}$ and wild-type

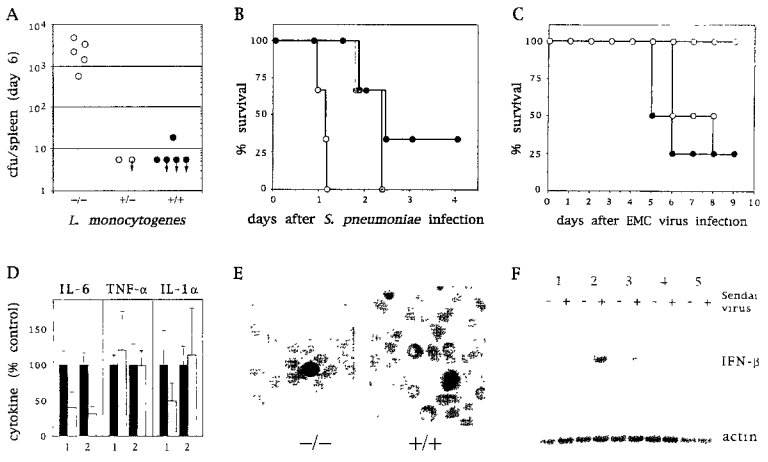

Figure 6. In Vivo Responses to Pathogens

Each circle represents an individual $p50^{-/-}$ (open circles), $p50^{+/-}$ (stippled circles), or wild-type (closed circles) animal.

(A) Defective clearance of L. monocytogenes. Mice were injected intraperitoneally with 5×10^3 cfu of L. monocytogenes and sacrificed 6 days after inoculation. Each circle represents the bacterial colony-forming unit (cfu) from the spleen homogenate of a single animal.

(B) Increased susceptibility to S. pneumoniae. Mice were injected intraperitoneally with 100 encapsulated S. pneumoniae bacteria, and the percentage of surviving mice was monitored over time. Of five similar experiments, using three littermates in each group, one experiment is shown.

(C) Increased resistance to infection with EMC virus. Mice were injected intraperitoneally with ~300 pfu of murine EMC virus, and the percentage of surviving mice was monitored over time. Of three similar experiments, using three $p50^{-/-}$ and four control littermates in each group, one experiment is shown.

(D) Cytokine production from LPS-stimulated resting peritoneal macrophages. Relative amounts (mean ± SD) of cytokines in 12 hr culture supernatants from LPS-stimulated resting peritoneal macrophages from individual animals were assayed from control (closed bars) and $p50^{-/-}$ (open bars) mice. IL-6, TNFα, and IL-1α production is shown with cytokine production from control resting peritoneal macrophages normalized to 100%. In experiment 1, five $p50^{-/-}$ and three control mice were used. In experiment 2, two $p50^{-/-}$ and five control mice were used.

(E) Representative blood smears from $p50^{-/-}$ and control mice 20 hr postinoculation with S. pneumoniae.

(F) Induction of IFNβ transcription in $p50^{-/-}$ (lanes 2 and 3) and control (lanes 1, 4, and 5) embryonic fibroblasts by Sendai virus. Embryonic fibroblasts were prepared from littermate embryos and either infected (plus) or mock infected (minus) with Sendai virus. Total cellular RNA was prepared 4 hr after infection, and 10 μg was used for Northern blot analysis. Induced IFNβ levels in arbitrary units (quantitated by PhosphorImager) were 29 ± 3.5 for $p50^{-/-}$ and 12 ± 4.3 for control embryonic fibroblasts.

mice. $p50^{-/-}$ mice died from overwhelming sepsis, apparently secondary to an inability to confine the infection locally to the peritoneum. In blood smears taken 20 hr after infection, control mice had no visible bacteria, while $p50^{-/-}$ mice had numerous encapsulated diplococci per high power field (Figure 6E). S. pneumoniae bacteria were seen extensively throughout all organ systems examined in moribund $p50^{-/-}$ mice.

The defect in control of S. pneumoniae did not extend to the gram-negative pathogens H. influenzae and E. coli K1, despite observed defects in both B cell and macrophage responses to LPS. The production of the early acute-phase reactants TNFα, IL-1, and IL-6 by macrophages stimulated with LPS is thought to involve regulation by NF-κB-binding sites in the promoters of these genes (Collart et al., 1990; Hiscott et al., 1993; Liebermann and Baltimore, 1990). When cytokine release was measured from $p50^{-/-}$ macrophages stimulated with LPS, TNFα and IL-1α release were normal, but IL-6 release was decreased severalfold relative to control macrophages (Figure 6D). Nevertheless, both control and $p50^{-/-}$ mice survived infectious challenges with 10^8 encapsulated type b H. influenzae and E. coli K1, although $p50^{-/-}$ mice did undergo a more sustained and elevated bacteremia following intraperitoneal inoculation with H. influenzae (data not shown). These findings suggest an important difference in the molecular mechanisms underlying sepsis caused by the major human pathogens. In the context of gram-negative infection, it appears that non-NF-κB-dependent activation of important defenses by LPS is sufficient to control progression of moderate disease. In contrast, the poor survival of $p50^{-/-}$ mice challenged with S. pneumoniae indicates that bacterial components other than LPS may activate additional antimicrobial defense cascades dependent upon NF-κB that are mobilized in the first 24 hr of disease.

Enhanced susceptibility to L. monocytogenes and S. pneumoniae contrasted with the greater resistance of $p50^{-/-}$ mice to infection with EMC virus, a cytopathic picornavirus that can lead to a fatal encephalopathy. When challenged intraperitoneally with ~200 pfu of EMC virus, $p50^{-/-}$ mice were surprisingly more resistant than control mice to lethal infection (Figure 6C). This resistance was relative; a 10-fold higher dose of EMC virus was lethal for $p50^{-/-}$ mice, although the progression to fatal encephalopathy was slower than in control mice. When infectious challenges were repeated with totally immunodeficient $RAG1^{-/-}$ mice, they succumbed 1–2 days earlier than control mice (data not shown), indicating that the protection against EMC virus in $p50^{-/-}$ mice was not due to a lack of immune responsiveness by lymphocytes.

The striking ability of $p50^{-/-}$ mice to show increased resistance to infection by EMC virus, in spite of the defects we had observed in specific effector lymphocyte and immune responses to bacteria, prompted us to look at possible antiviral responses that might be augmented in $p50^{-/-}$ mice. IFNβ is a type I IFN that is produced primarily by fibroblasts in response to viral infection. IFNβ mRNA is normally undetectable in primary fibroblasts, but high levels of this mRNA are induced when the cells are treated with virus (Goodbourn et al., 1985). This activation of IFNβ gene expression has been thought to involve viral induction of NF-κB that is subsequently bound to a virus-inducible transcriptional enhancer in the IFNβ promoter region (Lenardo et al., 1989; Maniatis et al., 1992). We examined the induction of IFNβ gene transcription by Sendai virus infection of embryonic fibroblasts. Interestingly, induced levels of IFNβ gene transcription were severalfold higher in virally infected $p50^{-/-}$ embryonic fibroblasts than in control fibroblasts (Figure 6F). This result suggested that the absence of p50 in embryonic fibroblasts augmented the antiviral IFNβ response and could explain the increased resistance to EMC virus.

Discussion

Targeted disruption of the *NFKB1* gene encoding the p105 precursor of the p50 subunit of NF-κB leads to apparently normal animals and, in particular, to unimpaired development of the immune system. The presence of a phenotypically intact immune system has allowed us to define the role of the transcription factor p50 in immune responses. Our results support a model in which NF-κB and other p50-containing complexes play no developmental role, but have evolved to be rapid response elements in both activation of lymphocytes and nonspecific immune responses to pathogens.

One difficulty in interpreting the results of gene targeting experiments involving a single member of a family of related proteins is that compensation, redundancy, or both by other family members may mask the actual importance of a particular family member. While a definitive assessment of the in vivo role of NF-κB must await characterization of mice that are lacking both p50 and p65 subunits, characterization of mutant mice lacking RelB provide support for the view that individual NF-κB/Rel family members play distinctive roles that cannot be compensated for by other family members (Weih et al., 1995 [this issue of *Cell*]). Mice lacking RelB exhibit marked disregulation of the hematopoietic system, including myeloid hyperplasia, splenomegaly due to extramedullary hematopoiesis, and multifocal inflammatory infiltrates (Weih et al., 1995). This split between a primary role in hematopoietic development for RelB and a primary role in functional immune responses for p50 is reminiscent of the distinction observed in Drosophila between the functions of the NF-κB/Rel proteins dorsal and Dif. Dorsal is involved in dorsal–ventral patterning in the Drosophila embryo (Steward, 1987). Dif, in contrast, is a rapid-response mediator of primitive immune responses to bacterial infection in Drosophila (Ip et al., 1993). It is likely that the ability of NF-κB/Rel proteins to play distinct roles in more primitive organisms has been maintained in vertebrates. This demarcation in function is likely to be one way in which the NF-κB/Rel family can coordinate many distinct input signals with so many discrete transcriptional outputs.

NF-κB Regulation of Lymphocyte Responses

Our results define a critical role for NF-κB in the control of terminal B cell differentiation rather than in the earlier

stages of B cell development, in which it has been thought to function in regulation of κ light chain gene expression. Mice lacking the p50 subunit of NF-κB have normal, mature B cells in which the frequency of κ light chain usage is normal. These mature B cells, however, exhibit focal defects in both mitogenic activation and basal and specific antibody production, indicating that NF-κB and other p50-containing complexes are key regulators of the activation and subsequent responses of mature B cells. We have also found that purified T cells lacking p50 proliferate poorly in response to T cell receptor and CD28 stimulation (data not shown), suggesting that NF-κB also plays a critical role in costimulation of T cell proliferation.

Our results suggest that tissue-specific regulation of NF-κB/Rel complexes in B cells may be essential to the control of terminal B cell differentiation. We previously described a developmental progression of NF-κB/Rel family member usage during different stages of B cell differentiation (Liou et al., 1994). We found that the pattern of NF-κB/Rel complexes seen in terminally differentiated B cells could be mimicked by long-term exposure of pre–B cell lines to LPS. This result prompted us to hypothesize a hierarchy among NF-κB/Rel complexes in B cells in which the emergence of constitutive NF-κB in mature B cells drives the appearance of subsequent NF-κB/Rel complexes that may be important in terminal B cell differentiation. This hypothesis may explain why we observe more pleiotropic defects in the functional responses of mature B cells than in other cell types lacking p50. In both B cells and fibroblasts, the primary NF-κB/Rel complex identified by EMSA was NF-κB p50–p65. Nevertheless, the absence of p50 substantially decreased the cytoplasmic levels of p65, Rel, and RelB in B cells, but had no effect on the levels of these family members in fibroblasts. This result implied that regulation of NF-κB/Rel family members is more tightly coupled to p50 in B cells than in fibroblasts. This tight coupling may reflect the relatively exclusive usage of p50 in heterodimeric NF-κB/Rel complexes in mature B cells. The presence of constitutive nuclear p50-containing NF-κB/Rel complexes that is observed uniquely in mature B cells may be linked to the ordered emergence of RelB and p52 in NF-κB/Rel complexes that occurs during terminal B cell differentiation. In other cell types that lack constitutive nuclear NF-κB, a hierarchy among NF-κB/Rel complexes may not exist, and either compensation or independent regulation of non-p50-containing NF-κB/Rel complexes in these cell types leads to little detectable change in the cytoplasmic levels of other family members in the absence of p50.

Dual Role of p50 in Immune Responses

The differences in the responses to infection with the three pathogens L. monocytogenes, S. pneumoniae, and EMC virus highlight the importance of NF-κB in regulating transcriptional responses to infection and indicate that p50-containing NF-κB/Rel complexes can play a dual role in either augmenting or decreasing specific immune responses. The surprising resistance to infection with EMC virus suggested that the absence of p50 was actually augmenting antiviral responses. We observed that virally infected fibroblasts that were lacking p50 had augmented induction of IFNβ transcription in comparison to control fibroblasts. This finding may reflect a dual role for p50 that has been suggested by several groups. While p50 forms potent transcriptional activators when heterodimerized with p65, Rel, or RelB, the ability of p50 homodimers to function as transcriptional activators has been more uncertain. While p50 homodimers have been reported to be transcriptionally active in cell-free transcription systems (Fujita et al., 1992; Kretzschmar et al., 1992), a number of studies strongly suggest that p50 homodimers lack a transcriptional activation domain and may be involved in transcriptional repression (Franzoso et al., 1992; Kang et al., 1992). While our results may be viewed as support for a model in which p50 homodimers are involved in postinduction repression of IFNβ gene transcription, they do not, as yet, allow us to distinguish between this model and the replacement of p50–p65 heterodimers in $p50^{-/-}$ fibroblasts with other more potent NF-κB/Rel transactivators at the κB site in the IFNβ gene. These findings, nevertheless, do demonstrate that the p50 subunit of NF-κB allows for down-regulation of transcriptional responses that may have important in vivo consequences in response to pathogens.

Regulation by the NF-κB/Rel Family

The in vivo consequences of gene targeting of the p50 subunit of NF-κB on the expression of genes for which extensive evidence for NF-κB regulation has been demonstrated reveal complex focal defects in NF-κB regulation that are difficult to predict based upon previous in vitro studies. For instance, expression of many genes, such as immunoglobulin κ light chain, class I MHC, and TNFα, was not affected by the absence of p50. In contrast, expression of genes such as IL-6 was reduced, whereas transcription of genes like IFNβ was augmented in the absence of p50. These results highlight our ignorance of the true in vivo regulators of transcription at particular κB sites in individual genes and suggest that many genes with κB sites are either not regulated by NF-κB or can be regulated by other NF-κB/Rel complexes in the absence of p50. In fact, our results with B cells suggest that p65–p65 or p65–Rel dimers may serve many of the functions in B cells that were previously ascribed to p50–p65. The unpredictable focal defects in NF-κB regulation that we observed in mice lacking p50 extended not only to the transcriptional regulation of individual genes but also to the induction of NF-κB/Rel complexes by different stimuli. We observed, for example, that while both LPS and antigen receptor stimulation are viewed as potent inducers of NF-κB/Rel complexes in wild-type B cells, the absence of p50-containing complexes revealed a differential ability to induce complexes involving p65 and Rel by antigen receptor stimulation, but not by LPS. The focal nature of the defects in transcription and induction that we have observed in $p50^{-/-}$ mice strongly implies that individual NF-κB/Rel complexes do play distinctive roles in the regulation of transcription of specific genes. It further suggests that we are beginning to reveal distinct modes of regulation in knockout mice, not easily assessed by other approaches, that play an important role in coupling distinct

input signals with transcriptional outputs through individual NF-κB/Rel family members.

The physiological role of the transcription factor p50 becomes clearer when we shift our focus from the regulation of individual genes to the regulation of gene networks controlling more complex in vivo processes. Here we find that p50 is critical for proliferative responses of lymphocytes, production of specific antibodies, and nonspecific immune responses to several pathogens, but is not essential for the development of the immune system. This lack of a developmental phenotype in $p50^{-/-}$ mice is striking when viewed in light of the complex disregulation of the hematopoietic system observed in mice lacking the more tissue-specifically expressed RelB family member (Weih et al., 1995). These results indicate that the ubiquitously expressed p50 subunit of NF-κB is likely to function solely as a rapid and pleiotropic mediator of different acute and specific immune responses and to play no role in development of the immune system.

Experimental Procedures

Construction of Targeting Vector

Phage clones of the *NFKB1* locus were isolated from a 129/Sv mouse liver DNA library (Stratagene). A NotI site was introduced by PCR mutagenesis at a position corresponding to residue 160 in exon 6 (amino acids 134–187 in the Rel homology domain of the p105 protein). This facilitated creation of an insertional targeting vector called pp50KO that, when linearized, contained 3.7 kb of 5′ flanking homologous DNA, a PGK–*neo* cassette (Tybulewicz et al., 1991) in the opposite transcriptional orientation in exon 6, 8.0 kb of 3′ flanking homologous DNA from the *NFKB1* gene, and a PGK–*tk* cassette (Tybulewicz et al., 1991) in the opposite transcriptional orientation, followed by the Bluescript II vector (Stratagene).

Generation of Mutant Mice

The linearized targeting vector pp50KO was electroporated into E14 embryonic stem cells and $G418^R$ colonies selected as described (Plump et al., 1992). Homologous recombinant clones were screened by Southern blotting using an external probe. Positive clones demonstrated a 9 kb recombinant band, in addition to the 10 kb wild-type band, in EcoRV-digested DNA. These clones were expanded and injected into C57BL6/J blastocysts. Resulting chimeric animals were backcrossed to C57BL6/J mice, and germline transmission was scored by coat color. $p50^{+/-}$ mice were identified by Southern blotting, and brother–sister mating was carried out to generate $p50^{-/-}$ mice.

Western Blots

Equal amounts of protein lysates from $p50^{-/-}$ and control tissues and cells were loaded onto individual lanes of protein gels. After transferral to nitrocellulose filters, the blots were stained with Ponceau S to confirm the uniformity of protein loading in each lane. Filters were then blocked with 2% nonfat milk–TBST (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 1 hr and incubated with specific antibody at a dilution of 1:1000 in 2% nonfat milk–TBST for 2 hr at room temperature. After three washes with TBST, filters were incubated with the secondary antibody (goat anti-rabbit IgG–horseradish peroxidase conjugate) for 1 hr, and antibody-reactive bands were revealed by chemiluminescent detection (ECL Western detection kit; Amersham International).

EMSA Assays

Preparation of nuclear and cytoplasmic extracts, EMSA, and antibody-inhibition assays to identify components of NF-κB/Rel complexes were performed as previously described (Liou et al., 1994). A loop oligonucleotide probe containing the class I MHC κB site was used in EMSA studies presented here, although similar results were obtained using an immunoglobulin κ κB site (Fujita et al., 1992).

Flow Cytometry

A FACScan flow cytometer and the Lysis II plotting program were used (Becton-Dickinson). First-step monoclonal antibody reagents used were R-PE-conjugated anti-Thy1.2 (30H.12), biotinylated anti-IgM (R6-60.2), biotinylated anti-κ light chain (R5-240), biotinylated anti-λ light chain (R26-46), biotinylated anti-H-$2K^b$ (AF6-88.5 clone), FITC-conjugated anti-CD45R/B220 (RA3-6B2), and biotinylated anti-I-A^b (AF6-120). Second-step reagents used were streptavidin–R-PE (Southern Biotechnology).

Cell Culture and Proliferation Assays

Cells were maintained in RPMI 1640 medium containing 10% defined fetal calf serum (Hyclone), penicillin–streptomycin, and 50 mM β-mercaptoethanol. B cells were isolated from spleen cell suspensions obtained by disrupting spleens between two frosted glass slides. Erythrocytes were removed from splenocytes by ammonium–chloride lysis. Resting B cells were purified from these crude spleen populations by one round of complement lysis with a Thy1.2 antibody to remove T cells. The remaining cells were then fractionated through Percoll gradients (Coligan et al., 1993) to isolate small resting B cells, contained between 66% and 70% Percoll. These cells were collected, washed extensively, and incubated in medium for stimulation with anti-IgM or LPS. Proliferation assays were performed by plating out 10^5 purified B cells in triplicate for each condition in 96-well plates containing 200 μl of media. After 60 hr, 1 μCi of [methyl-^{3}H]thymidine was added to each well for 12 hr, and [^{3}H]thymidine incorporation into DNA was quantitated by scintillation counter.

Immunoglobulin Isotype Analysis

Sera were prepared from 6-week-old $p50^{-/-}$ and control $p50^{+/-}$ sex-matched littermates. Serum levels in $p50^{+/-}$ and wild-type littermates had previously been determined to be indistinguishable. Immunoglobulin isotypes were quantitatively determined against isotype standards using a sandwich enzyme-linked immunosorbent assay (ELISA) employing anti-IgE monoclonal antibodies (PharMingen) for IgE and using a pan-specific capture antibody and isotype-specific antibodies conjugated to horseradish peroxidase (Southern Biotechnology) for the other isotypes.

T Cell-Dependent Immune Response

Littermates were injected intraperitoneally with alum-precipitated NP_{15}-CG (100 μg per mouse), and sera were collected at day 6 for the IgM response and at day 15 for the IgG response. Immunoglobulin specific to NP was measured using a sandwich ELISA in which NP-specific immunoglobulin was captured on plates coated with NP-BSA and was analyzed for immunoglobulin isotype.

Cytokine Release from Peritoneal Macrophages

Resting peritoneal macrophages were isolated by peritoneal lavage with 5 ml of ice-cold PBS. Isolated cells were washed and ~10^6 cells plated per condition in 24-well plates. After several hours at 37°C, nonadherent cells were removed by two washes with warm PBS, and adherent cells were incubated in 0.5 ml of medium containing LPS. After removal of supernatants to measure cytokine levels, adherent cells were washed in PBS and total protein determined by Coomassie protein assay (Pierce) for normalization of cytokine release to numbers of adherent cells. Cytokine levels in tissue culture supernatants were measured using sandwich ELISAs as described (Sheehan et al., 1989). For IL-1α, anti-mouse IL-1α (Genzyme) and polyclonal rabbit anti-mouse IL-1α were used. For IL-6, anti-murine IL-6 (MP5-20F3) and biotinylated anti-murine IL-6 (MP5-32C11) were used.

Pathogen Challenges

L. monocytogenes (43251) and murine EMC virus (VR-129B) were obtained from the ATCC. S. pneumoniae type 2 strain AII, H. influenzae type b Eagan, and E. coli K1 were maintained at the Laboratory of Molecular Infectious Diseases at The Rockefeller University.

Induction of IFNβ mRNA and Northern Blot Analysis

For induction by Sendai virus (SPAFAS), embryonic fibroblasts were washed twice in PBS and then incubated for 2 hr in serum-free medium containing ~300 hemagglutinating units per milliliter of virus. Virus was removed by washing the cells twice with PBS, and then normal

medium was added for a further 2 hr before RNA preparation. Total cellular RNA was prepared using TRI-REAGENT (Molecular Research Center). A mouse IFNβ probe containing the first 500 bp of coding sequence was hybridized against 10 μg of RNA. A γ-actin probe (Scott et al., 1993) was later used to reprobe the same blot. Levels of IFNβ RNA were quantitated by PhosphorImager and normalized to γ-actin.

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