IkBε-Deficient Mice: Reduction of One T Cell Precursor Subspecies and Enhanced Ig Isotype Switching and Cytokine Synthesis

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IκBe-Deficient Mice: Reduction of One T Cell Precursor Subspecies and Enhanced Ig Isotype Switching and Cytokine Synthesis

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Three major inhibitors of the NF-κB/Rel family of transcription factors, IκBα, IκBβ, and IκBε, have been described. To examine the in vivo role of the most recently discovered member of the IκB family, IκBε, we generated a null allele of the murine IκBε gene by replacement of all coding sequences with nlslacZ. Unlike IκBα nullizygous mice, mice lacking IκBε are viable, fertile, and indistinguishable from wild-type animals in appearance and histology. Analysis of β-galactosidase expression pattern revealed that IκBε is mainly expressed in T cells in the thymus, spleen, and lymph nodes. Flow cytometric analysis of immune cell populations from the bone marrow, thymus, spleen, and lymph nodes did not show any specific differences between the wild-type and the mutant mice, with the exception of a reproducible 50% reduction of the CD44+CD25+ T cell subspecies. The IκBε-null mice present constitutive up-regulation of IgM and IgG1 Ig isotypes together with a further increased synthesis of these two isotypes after immunization against T cell-dependent or independent Ags. The failure of observable augmentation of constitutive nuclear NF-κB/Rel-binding activity is probably due to compensatory mechanisms involving IκBα and IκBθ, which are up-regulated in several organs. RNase-mapping analysis indicated that IL-1α, IL-1β, IL-1Ra, and IL-6 mRNA levels are constitutively elevated in thioglycolate-elicited IκBε-null macrophages in contrast to GM-CSF, G-CSF, and IFN-γ, which remain undetectable. The Journal of Immunology, 1999, 163: 5994–6005.

The NF-κB family of transcription factors controls the expression of a wide array of genes, such as genes encoding cytokines (IL-1α and β, IL-2, IL-6, IL-8, TNF-α, GM-CSF, etc.), cytokine receptors, adhesion proteins, immunoregulatory molecules (MHC class I, etc.), antiapoptotic and acute-phase response proteins, in addition to viral promoters, like CMV or HIV (1–3), and thus plays a key role in immune and inflammatory processes, as evidenced by recent knockout analyses of NF-κB genes in mice (4–7). NF-κB molecules consist of homo- and heterodimeric combinations of proteins of the same family, which comprises five members in mammals, p50, p52, p65 or RelA, c-Rel, and RelB (1, 2, 6). These proteins share a 300-aa NH2-terminal Rel homology domain, involved in DNA binding, dimerization, and nuclear localization (8). In contrast to p65, c-Rel, or RelB, which contain a C-terminal transactivation domain, p50 and p52 are synthesized as precursor molecules, p105 and p100, respectively, which are solely cytoplasmic and do not bind DNA. NF-κB/Rel complexes are constitutively active in the nuclei of mature T and B lymphocytes as well as in neurons of certain regions of the brain (8, 9). In the vast majority of cells, however, NF-κB/Rel complexes are sequestered in the cytoplasm by interaction with inhibitory molecules, called IκBα, which mask the NF-κB nucleus localization and DNA binding domains (6, 8, 10). Multiple stimuli, as varied as exposure to proinflammatory cytokines like TNF or IL-1, viral infection, or chemical agents such as phorbol esters, UV irradiation, and phosphatase inhibitors, converge via distinct signaling pathways to activate NF-κB by phosphorylation of IκBα, which leads to IκB ubiquitination and subsequent proteolytic degradation by the proteasome (2). Free NF-κB/Rel complexes then migrate to the nucleus and activate transcription of their target genes.

The IκB family comprises three major members, IκBα, IκBβ, and IκBε, defined by the presence of six ankyrin repeats, a 33-aa motif that mediates protein-protein interactions (10). IκBα and IκBβ were originally purified from cytosolic fractions as NF-κB-associated inhibitory activities (11, 12) and their genes were cloned soon after (13, 14). IκBε was recently identified in yeast two-hybrid screens on the basis of protein-protein interactions with p52 (15), p50 (16), or p65 (17). Despite their extensive structural

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similarities, IκBeα, IκBβ, and IκBe behave differently in response to external stimuli. Generally, IκBα is rapidly degraded and re-synthesized, whereas IκBβ and IκBe are degraded with a slower kinetics to gradually reappear (15, 18). Newly synthesized IκBα has been shown to be responsible for postinduction repression of NF-κB activity by entering the nucleus and removing NF-κB/Rel complexes from target promoters (19). The nuclear export sequence of IκBα, which is exposed upon binding to NF-κB, then drives NF-κB/IκBα complexes out of the nucleus (20). Newly synthesized, hypophosphorylated forms of IκBβ interact with the NF-κB/Rel complexes bound on target promoters, without disrupting the DNA-binding interaction, thereby leading to a sustained NF-κB response by protecting the NF-κB molecules from inhibition by nuclear IκBα (21). IκBe is exclusively cytoplasmic and has been found specifically associated with c-Rel, p65 homodimers, or p65/c-Rel heterodimers in cell extracts (15, 16). It is unclear whether different IκB family members have distinct roles or redundant functions in vivo. To address this question, knockout and knockin studies at the IκBα locus together with gain of function analysis of IκBα and IκBβ, under the control of T cell-specific promoters, have recently been undertaken (22–26). IκBα knockout mice die by 7 to 8 days after birth and exhibit extensive granulopoiesis, acute running, and abnormal skin formation (22, 23). Interestingly, the absence of IκBα induces the up-regulation of IκBe (15) and renders mouse embryonic fibroblasts (MEFs) unable to terminate the NF-κB activation after TNF treatment (22, 23). These results confirm the essential role of IκBα in postinduction repression and demonstrate that this function cannot be substituted for by IκBβ or IκBe. Mice containing a knockin of the IκBβ coding region at the IκBα locus are, on the contrary, totally viable and show no difference in constitutive or induced NF-κB response compared with wild-type mice (24). These data indicate that IκBα and IκBβ share enough biochemical properties to substitute one for another and that their specific in vivo roles arise from their distinct expression patterns.

To gain insight into the physiological role of IκBe, we created IκBe null-mutant mice by targeted IκBe gene replacement with nlslacZ. Such mice develop normally and do not exhibit morphological defects. Analysis of β-galactosidase activity in these mice reveals that, within the immune system, IκBe is expressed mainly in thymocytes. IκBe-deficient animals have normal mature hemopoietic cells, in spite of a 50% reduction in the number of CD44+CD25+ precursor T cells. They respond normally to mitogenic stimuli and bacterial challenge, but have an altered basal and Ag-specific Ig production. The expression of a number of cytokine genes is specifically up-regulated in IκBe-null mice, but remarkably not those known to be regulated by p65 and/or c-Rel dimers, such as GM-CSF. The mild phenotype observed for IκBα and IκBβ encoding regions. A 2.5-kb SauI-SmaI fragment containing the 5′ region of the murine IκBe gene was subcloned into a derivative of p210lacZ (kind gift of S. Tajbakhsh, Institut Pasteur, Paris, France), containing a 5′-end IRES, to constitute the final targeting construct.

Materials and Methods

Design of the targeting construct

A A DASHII library carrying DNA fragments from a partial Sma3A digest of mouse male 129/SV genomic DNA was screened with the entire human IκBe cDNA (15). Five bacteriophages spanning a 17.5-kb region encompassing the whole IκBe gene were isolated, and their inserts were subcloned into the BamHI site of Bluescript SK+ (Stratagene, La Jolla, CA). Restriction mapping and partial sequencing were used to localize IκBe encoding regions. A 2.5-kb SauI-SmaI fragment containing the 5′ region of the murine IκBe gene was subcloned into a derivative of p210lacZ (kind gift of S. Tajbakhsh, Institut Pasteur, Paris, France), containing a XbaI site inserted upstream of the NcoI site, giving rise to p5′210lacZ, a plasmid in which the 5′ region of IκBe drives the expression of the lacZ gene with an associated nuclear localization sequence (nlslacZ). A 1.8-kb Asp718, HindIII fragment isolated from pPN5 (27), which included the HSV-thymidine kinase gene (HSVtk) under the control of the phosphoglycerate kinase (pck) promoter together with the pgk poly(A) was introduced upstream of the 5′ region of IκBe, to generate pPN5′210lacZ. A 6.6-kb BamHI fragment enclosing the 3′ region of the murine IκBe gene was placed upstream of the tk promoter-enhancer driving expression of the diphtheria toxin A-fragment gene (DTA) devoid of poly(A) sequences (ptk-DTA, kind gift of S. Tajbakhsh), generating p3′dtk-DTA. A 5.5-kb XhoI fragment from p210Z (kind gift of S. Tajbakhsh, Institut Pasteur, Paris, France), containing nlslacZ, followed by the encephalomyocarditis virus internal ribosome entry site (IRES) upstream of the neomycin-resistance gene (neo), was integrated into the Xhol-EcorV site of p3′dtk-DTA to create p210Z′ntk-DTA. Finally, an 11.1-kb XbaI fragment isolated from pPN5′210lacZ, containing hsttk, the 5′ region of IκBe and nsls lacZ, was subcloned into the XbaI site of p210Z′ntk-DTA upstream of the IRES, to constitute the final targeting construct.

ES cell transfection and selection

H1M ES cells (courtesy of David Melton, Edinburgh, U.K.) were grown on primary fibroblast feeder layers in the presence of LIF (104 U/ml). A total of 105 cells were electroporated with 10 μg of Norl-linearized targeting construct in 4-mm cuvettes with a Eurogentec Cellject electroporator at 1,000 V/m. Cells were diluted into 40 ml of prewarmed complete medium, plated, and left to recover for 48 h before addition of 0.3 mg/ml G418 (Life Technologies, Cergy, France) or without gancyclovir at 5 μM. Once established, clones were maintained in complete medium without selection.

Generation and characterization of knockout mice

Two independently targeted ES cell clones were injected into C57BL/6 blastocysts, which were subsequently reimplanted into pseudopregnant C57BL/6 × CBA females. Resulting chimeras were mated to C57BL/6 × DBA/2 mice, and heterozygous offspring for the targeted allele were interbred. The two independent IκBe-null mouse lines generated were found to be identical in all subsequent experiments. Mice were kept in clean housing conditions in a level 2 barrier animal house facility. When specified, mice were analyzed between 6 and 12 wk of age.

For Southern blot analysis, genomic DNA was isolated from tail biopsies according to Laird et al. (28). A total of 10 μg of DNA was digested with XbaI and BamHI, separated by electrophoresis on 0.9% agarose gel, transferred onto nylon membrane (Amer sham Pharmacia Biotech, Amer sham, U.K.), and hybridized with a random-primed 32P-labeled 5′ external probe (29).

For genotyping by PCR, tail biopsies were lysed in a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 1 mg/ml proteinase K (Boehringer Mannheim, Meylan, France) at 55°C for at least 3 h. After centrifugation at 14,000 rpm, the supernatant was recovered, heat denaturated at 100°C for 10 min, and used directly in PCR reaction. The forward primer is contained within the 5′ external probe used for Southern analysis (5′-cgcctgacgacctggaccac-3′). Two reverse primers were used: one within the first exon of the IκBe gene for primer 1 (5′-cgcctgctcttcatgcctatctctct-3′), which amplifies the wild-type allele, and a second within the lacZ gene (5′-cgcctgacgacctggaccac-3′), which amplifies the mutant allele. PCR reaction mix included 0.25 μM of each of the three primers, 1 mM MgCl2, 0.2 mM dNTP, and 1 U of Tag DNA polymerase (Promega, Charbonnières, France). Denaturated samples were subjected to 35 cycles of amplification in a PTC-200 thermal cycler (MJ Research, Cambridge, MA), cycling parameters being the following: 94°C denaturation for 40 s, followed by a combined annealing and polymerization step at 72°C for 4 min. After an extra step at 72°C for 10 min, 10 μl of the reaction was analyzed on a 2.4% agarose gel (containing one-third of NuSieve GTG agarose (FMC) and two-thirds of standard agarose from Life Technologies). Amplified products were detected by ethidium bromide staining.

RNA isolation and Northern blot analysis

Total RNA was isolated from organs or cells using the TRIZol Reagent (Life Technologies), according to the manufacturer’s instructions. The RNA pellet was resuspended in nuclelease-free H2O. A total of 30 μg of total RNA was separated by electrophoresis on 1% denaturing formaldehyde agarose gel, transferred onto nylon membrane (Amer sham Pharmacia Bio tech), and hybridized with a 32P-labeled probe corresponding to full-length human IκBe cDNA.
Western blot analysis and Abs

Whole-cell extracts or cytoplasmic extracts were prepared as previously described (9), and proteins were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) or Immobilon membranes (Millipore, Bedford, MA). Immunoblots were incubated with rabbit polyclonal Abs at 1/1000 dilution (or 1/200 dilution for commercial Abs) and revealed with the Pierce (Rockford, IL) enhanced chemiluminescence system, as recommended by the manufacturer. Sera raised against murine p50 (1263), p65 (1226), p52 (1267), and c-rel (1051) were kind gifts of N. Rice (Frederick, MD); serum against RelB was kindly provided by R. Bravo (Princeton, NJ); serum against IκBα was a generous gift of R. Weil (Institut Pasteur, Paris, France); and sera against IκBα (C-21) and IκBε (M-121) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments

Single-cell suspensions of splenocytes or T cells, isolated from thymic suspensions by purification on MACS CD90 (Thy-1.2) MicroBeads (Miltenyi Biotec., Auburn, CA), were cultured at 37°C, 5% CO2 in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 U/ml penicillin/streptomycin, 2 mM glutamine, and 50 µM 2-ME (all from Life Technologies). Peritoneal macrophages were cultured under the same conditions, but in the absence of 2-ME. LPS from Salmonella abortus equi (Sigma, Saint Quentin Fallavier, France), ALLN (Sigma, Saint Quentin Fallavier, France), according to Fiering et al. (30). Cells were then surface stained either with mAbs coupled to fluorescein or PE, or with biotinylated mAbs (PharMingen, San Diego, CA; Caltag, San Francisco, CA), were prepared and bandshift assays were performed as previously described (9), using the Pierce (Rockford, IL) enhanced chemiluminescence system, as recommended by the manufacturer. Sera raised against murine p50 (1263), p65 (1226), p52 (1267), and c-rel (1051) were kind gifts of N. Rice (Frederick, MD); serum against RelB was kindly provided by R. Bravo (Princeton, NJ); serum against IκBα was a generous gift of R. Weil (Institut Pasteur, Paris, France); and sera against IκBα (C-21) and IκBε (M-121) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Histological analysis and β-galactosidase in situ staining

Organs were fixed for 20–30 min in 4% paraformaldehyde in PBS, incubated in 15% sucrose, 1× PBS for 15 h at 4°C, frozen, and embedded in Tissue-Tek OCT compound (Miles-Bayer). Sections (20 µm) were cut using a cryostat (Leica) and collected on SuperFrost Plus slides (Menzel-Glaser, Germany). Sections were X-gal stained, as previously described (9), and counterstained with eosin. Dehydrated sections were mounted using EUKITT and photographed on a Nikon microscope equipped with a camera, using Kodak Ektachrome 64T film.

Flow cytometric analysis

Single-cell suspensions from thymus, bone marrow, spleen, and lymph nodes were surface stained either with mAbs coupled to fluorescein or PE, or with biotinylated mAbs (PharMingen, San Diego, CA; Caltag, San Francisco, CA), followed by tricolor-streptavidin (Caltag). Viable cells (propidium iodide negative) were then analyzed using a FACSscan fluorocytometer (Becton Dickinson, Mountain View, CA). The mAbs used were anti-CD3ε (145-2C11), anti-CD4 (L3T4), anti-CD8 (Ly-3.2 and Ly-2/53-5), anti-CD11b/Mac1 (M1/70), anti-CD11c (HL3), anti-CD19 (1D3), anti-CD24/HSAA (M1/69), anti-CD25 (7D4), anti-CD44 (5S), anti-CD45 (Ly24/Pgp-1), anti-CD45R/B220 (RA3-6B2), anti-Gr1 (Ly-6G/RB6-8C5), anti-B220 (Ly-5/5163C), Ter 119, and anti-IgM (R6-60.2).

For identification of β-galactosidase-expressing cells, lymphocytes isolated by gradient centrifugation through Ficoll-Isopaque (Amersham Pharmacia Biotech) were first stained with a vital fluorogenic substrate, fluoresein-di-β-D-galactopyranoside (FDA; Molecular Probes, Interchim, Montluçon, France), according to Fiering et al. (30). Cells were then washed once before additional immunolabeling and analysis by flow cytometry. Fluorescein background was determined by performing the β-galactosidase assay on lymphocytes isolated in parallel from a wild-type control mouse.

Proliferation assay

Splenocytes (2 × 10^5/ml) were incubated in the presence or absence of various concentrations of anti-CD3ε (145-2C11), anti-IgM Abs (PharMingen), or LPS. Cells were pulsed with [3H]thymidine (1 µCi/ml; Amersham Pharmacia Biotech) and transferred after 72-h culture onto glass filter mats, and radioactive incorporation was measured with a β-scintillation counter.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from whole organs or T cells, purified by MACS CD90 (Thy-1.2) MicroBeads, were prepared and bandshift assays were performed as previously described (9), using the κB site derived from the promoter of the MHC class I H-2 K^d gene as a probe.

Ig isotype analysis

Sera were prepared from 5- to 6 wk old IκBε^-/- and control wild-type sex-matched littermates. Ig isotypes were quantitatively determined against isotype standards using a sandwich ELISA with a pan-specific capture Ab (Southern Biotechnology Associates, Birmingham, AL) and isotype-specific Abs conjugated to alkaline-phosphatase (Southern Biotechnology Associates).

Immunization and T cell-dependent and independent humoral immune responses

Sex-matched animals 5 wk old were immunized with p.i. injection of either 100 µg of KLH coupled to DNP precipitated in alum (T cell-dependent responses) or 50 µg of LPS coupled to DNP (T cell-independent responses). Serum samples were collected from the mice before immunization and at 7-day intervals after immunization for a period of 3 wk. Levels of DNP-specific Ig isotype were determined by ELISA using DNP-BSA (17:1) as a capture agent and goat anti-mouse isotype-specific Abs directly conjugated to alkaline-phosphatase (Southern Biotechnology Associates).

RNase protection assay

Animals 12 wk old were injected i.p. with 2 ml of resazurin-thioglycollate (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). After 5 days, peritoneal macrophages were collected from pools of five to six mice and incubated for 8 h at 37°C with or without LPS. Total RNA was then isolated from adherent macrophage cells, as described above, and ribonuclease protection assay was performed with 6 µg of total RNA and the RiboQuant In Vitro Transcription and RPA kits (PharMingen). The murine cytokine sets mCK-2 and mCK-4 (PharMingen) were used to obtain radiolabeled antisense RNA probes for IL-1α, IL-1β, IL-1Ra, IL-3, IL-6, IL-7, IL-11, IL-12p35, IL-12p40, IL-10, IFN-γ, LIF, macrophage migration inhibitory factor, G-CSF, GM-CSF, M-CSF, stem cell factor, L32, and GAPDH. The RNA duplexes were analyzed by electrophoresis on 5% polyacrylamide/8 M urea gels, which were then dried and subjected to autoradiography. Bands were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Pathogen challenges

Mice 15 wk old were challenged via intranasal injection under ethyl ether anesthesia to 20 µl (1 × 10^3 bacteria) of a suspension of Shigella flexneri strain M90T-GFP, established by M. Rathman (unpublished) and grown as described in Phalipon et al. (31). Mice 12 wk old were inoculated i.v. with 5 × 10^7 bacteria from Listeria monocytogenes EGD strain (32). At the indicated time after inoculation, lungs were collected from S. flexneri-infected mice, or spleen, liver, and brain were removed from L. monocytogenes-infected mice. Each organ was homogenized with an Ultra-turrax blender in sterile saline solution. For each organ, bacteria were titrated on agar plates or BHI agar. For each plate, 1 ml of serial dilutions plated on Congo red agar (infection by S. flexneri) or BHI agar (infection by L. monocytogenes) and incubated at 37°C. Results were expressed as the number of CFU per gram of lung tissue for S. flexneri and as the logarithm of CFU per organ for L. monocytogenes infection.

Results

Generation of IκBε-deficient mouse

To inactivate the murine IκBε gene by homologous recombination in ES cells, we cloned a 17.5-kb genomic region encompassing the IκBε gene from a 129/SV library, using the human IκBε cDNA as a probe (15). The targeting construct was designed (Fig. 1A) to replace the entire coding sequence of IκBε, starting from the ATG, by nlslacZ to follow the expression pattern of IκBε in vivo (33). The construct contains a promoterless neo gene (34), positioned downstream of nlslacZ, and whose translation is dependent on the presence of an upstream IRES (35). Hsv-tk and DTA were placed at each end of the construct to achieve a double-negative selection (36, 37). We checked that IκBε mRNA was detectable in ES cells, even if it was at very low levels compared with those of IκBβ or IκBα, this latter constituting the major IκB species in these cells (data not shown). By transient transfection experiments into ES cells, we also verified that the 2.5-kb 5' fragment used in the targeting construct was unable to drive nlslacZ gene expression by itself (data not shown). Following electroporation of the targeting vector into HM1 ES cells, G418-resistant colonies obtained in the presence or absence of gancyclovir were selected and screened by
FIGURE 1. Generation of IkBe-deficient mice. A, Schematic representation of the wild-type (WT) and mutated (MT) genomic IkBe loci together with the targeting vector. Restriction enzyme sites (B, BamHI; S, SacI; Sm, SmaI; X, XbaI) and the probe for Southern analysis are indicated. The precise position of the exons in the genomic DNA is unknown. Homologous recombination of WT DNA with the targeting construct results in the replacement of all IkBe coding sequences with nlslacZiresneo sequences. B, Southern blot analysis of DNA preparations from tails of adult mice from heterozygote matings. The three genotypic categories obtained correspond to wild-type (lane 1), heterozygote (lanes 2 and 3), and homozygote (lanes 4 and 5). Digestion of genomic DNA with BamHI plus XbaI, followed by hybridization with the 5’ external probe depicted above, generates a 3.4-kb fragment for the wild-type (WT) allele and a 6.2-kb fragment for the mutated (MT) allele. C, PCR analysis of the DNA samples used in B. Design of primers allows the amplification of a 247-bp fragment for the wild-type (WT) allele and a 550-bp fragment for the mutated (MT) allele. D, Northern blot analysis of total RNA from wild-type, heterozygous, and homozygous mutant IkBe mice. Total RNA (30 µg) extracted from the thymus (lanes 1–4), spleen (lanes 5–8), ovary (lanes 9–11), or lung (lanes 12–14) was electrophoresed and transferred to nylon membrane. Top panel, Hybridization with full-length human IkBe cDNA. Autoradiography for thymus and spleen has been exposed 2.5-fold less than that for ovary and lung. Bottom panel, The same filter was rehybridized with a S26 probe (68). Lanes 1, 5, 9, and 12, Wild-type; lanes 4, 8, and 11, heterozygotes; lanes 2, 3, 6, 7, 10, 13, and 14, homozygous mutants. E, Western blot analysis of protein extracts from wild-type, heterozygous, and homozygous mutant IkBe mice. Total extracts from spleen (200 µg) (top panel) or thymus (100 µg) (bottom panel) were examined by immunoblot analysis with a polyclonal Ab directed against IkBe. Top panel (spleen), lane 1, wild type; lanes 2 and 3, heterozygotes; lanes 4, 5, and 6, homozygous mutants. Bottom panel (thymus), lanes 1 and 5, wild type; lanes 2 and 4, heterozygotes; lanes 3, 6, 7, 8, and 9, homozygous mutants. Densitometry scanning of bands in bottom panel, thymus, lane 5 indicates a more intense signal compared with that observed for lanes 3 and 4.
Southern blot analysis. Of 42 recombinant ES clones examined, 9 were positive for homologous recombination and 1 of these 9 clones harbored an additional insertion in the genome, as evidenced by probing with a lacZ fragment (data not shown). Selection in the presence or absence of gancyclovir yielded the same percentage of homologous recombinant clones, indicating that DTA alone was sufficient for negative selection. Two correctly targeted clones from two independent electroporations were injected into C57BL/6 blastocysts and gave rise to chimeric mice that displayed 100% color chimerism and that all transmitted the recombinant allele to their progeny (with one exhibiting 50% germline transmission). Two independent IκBe null mice were thus established, analyzed in parallel, and found to be indistinguishable in all subsequent experiments. Intercrossing heterozygous animals, which displayed no obvious phenotype, generated IκBe−/+, IκBe+/−, and IκBe−/− mice, identified by Southern blot and PCR analysis (Fig. 1 B and C). IκBe expression was abolished in homozygous mutants, as determined by Northern blotting with total RNA purified from several organs (Fig. 1 D) and Western blot analysis of spleen and thymus total protein extracts (Fig. 1 E). Intermediate levels of IκBe mRNA or protein were observed in every heterozygote organ examined (Fig. 1 D, lanes 4, 8, and 11, and Fig. 1 E, lanes 2 and 3; spleen; and lanes 2 and 4, thymus), indicating that both IκBe alleles are functional in wild-type mice. IκBe−/− mice were born at the expected Mendelian segregation ratios and grew normally. No evident morphological or behavioral abnormalities were detected. IκBe-null mice did not show any gross macroscopic alterations either, as determined by detailed histopathological analysis, and their skeleton appeared indistinguishable from that of wild-type mice by x-ray irradiation and Alcian blue/Alizarin red staining (data not shown).

Normal histology of lymphoid organs of IκBe-deficient mice

Examination of tissue sections from thymus, spleen, or lymph nodes of wild-type (+/+), or homozygous mutant (−/−) mice did not display any significant differences (Fig. 2). In situ staining of these sections by X-gal, a colorimetric substrate for β-galactosidase, was used to elucidate the expression pattern of IκBe in these organs, the nlslacZ gene having been knocked in to the IκBe locus and thus placed under the control of the endogenous IκBe regulatory sequences. X-gal staining revealed that the major site of IκBe expression is the lymph node, and especially the medulla. LacZ expression is scattered throughout the thymus and restricted to a few cells in the spleen (Fig. 2). Further characterization of the β-galactosidase-expressing cells was performed by flow cytometry with simultaneous detection of surface Ags and fluorescent staining of β-galactosidase using the FDG fluorogenic vital substrate (38). Results presented in Table I show that the highest number of FDG+ cells is located within the lymph node (50%) and decreasingly in the thymus (30%) and the spleen (5%), corroborating the in situ staining data. Interestingly, in each organ, T cells constitute the major FDG+ species. Some B cells (between 4 and 15% of FDG+) lymphocytes also exhibit β-galactosidase activity in the two secondary lymphoid organs examined. Immunolabeling of lymph node cells with Mac1, GR1, or CD11c did not reveal any specific FDG+ population, the number of immunolabeled and

Table I. Identification of lymphocyte subsets expressing IκBe by FACS analysis of FDG-stained and immunolabeled lymphocytes from IκBe-mutant mice

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<td>70</td>
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<td>(95%)</td>
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<td>Lymph nodes</td>
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<tr>
<td>FDG+</td>
<td>92</td>
<td>65</td>
<td>26</td>
<td>–</td>
<td>4</td>
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<td>(50%)</td>
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<tr>
<td>FDG−</td>
<td>56</td>
<td>31</td>
<td>18</td>
<td>–</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>(50%)</td>
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*Percentages of cells, isolated from the thymus, spleen, or lymph nodes of IκBe-mutant heterozygote mice, expressing β-galactosidase activity vs. CD3, CD4, CD8, B220 immunolabeling are presented. In brackets are indicated percentages of FDG+ or FDG− cells vs total lymphocyte population. Percentages represent the means of four heterozygote mutant mice. Similar values were obtained for homozygous mutant mice (data not shown).
FDG+ cells being 1.5%, 4%, and 1%, respectively (data not shown).

Normal mature hemopoietic cell populations and diminution of one T cell precursor subset in IκBε-deficient mice

We next investigated whether hemopoietic cell populations were normal in IκBε-null mice. Results from flow cytometric analyses of bone marrow, thymic, and splenic cell populations are presented in Fig. 3. No difference between the wild-type (+/+ ) and the homozygous mutant (−/−) mice was observed in the expression of B cell Ags (B220, IgM), erythroid cell Ag (Ter119), granulocyte Ag (GR1), and macrophage Ag (Mac1) in the bone marrow (A), or B cell Ags (B220, IgM) and T cell markers (CD3) in the spleen (C). We also tested surface markers for precursor B cells according to the classification of Hardy (39), such as CD43, HSA, CD19, and macrophage Ag (Mac1) in the bone marrow, markers for dendritic cells like CD11c in the spleen, and markers for granulocytes, GR1, and macrophages, Mac1, in the lymph nodes (data not shown). No significant differences were detected between wild-type and mutant animals. In the thymus (B), the percentage of CD4, CD8 double or simple positive cells are identical in both types of mice. Remarkably, when we examined the expression of CD44 and CD25 in the CD4−/CD8− population, we observed a 50% fold reduction in the percentage of CD44+CD25+ cells in IκBε-null mice compared with control wild-type littermates, the other populations being normal. Peripheral blood analysis of IκBε-deficient mice revealed a standard cell count for every cell population, other hemogram parameters being average (data not shown). Overall, these results establish the presence of normal numbers of mature B cells, T cells, erythroid cells, dendritic cells, granulocytes, and macrophages in IκBε mutants even though one precursor T cell subspecies is diminished.

Normal proliferation of splenocytes from IκBε-deficient mice in response to mitogenic stimuli

We next investigated whether splenocytes from wild-type (+/+), heterozygote (+/−), or IκBε-null (−/−) mice responded normally to various mitogens known to induce B and/or T cell proliferation via distinct signaling pathways. Results presented in Fig. 4 reveal no significant difference in the proliferative response of the splenocytes derived from these three genotypes to anti-CD3 (A), LPS (B), or anti-IgM (C), albeit large variations were observed among individuals.

We then asked whether the mild phenotype observed for IκBε-deficient mice could be due to compensatory mechanisms involving up-regulation of other IκB family members. We thus assessed the levels of other IκB molecules in the spleen of IκBε-null (−/−)
mice. A representative Western blot analysis of total splenic extracts of wild-type (+/+) and IκBε-null (−/−) mice is presented in Fig. 5 (n = 4). In IκBε-deficient mice, increased levels of IκBα and IκBβ were observed, whereas expression of p105 and p100 was not significantly affected (A). As expected, IκBβ, found in wild-type cells, was absent in the homozygous mutant cells (A, upper panel). Since the synthesis of three members of the NF-κB family, p105, p100, and c-Rel, is directly controlled by NF-κB itself (2, 6), we also looked at the expression of p50, p65, c-Rel, and RelB (B). The steady state levels of all these molecules were similar in IκBε-deficient and wild-type mice. This latter result together with the observed up-regulation of IκBα and IκBβ suggests that constitutive nuclear NF-κB activity may be unchanged in IκBε-deficient mice. This was shown to be the case when we performed bandshift assays with nuclear extracts from the spleen or resident macrophages of homozygous mutant or control wild-type littermate mice (data not shown).

Normal TNF response of T cells from IκBε-deficient mice

Since IκBε is preferentially expressed in T cells (see above), we examined how mutant T cells responded to TNF treatment. Thymocytes purified from wild-type (+/+) (lanes 1–5), heterozygote (+/−) (lanes 6–10), or IκBε-deficient (−/−) (lanes 11–15) mice were incubated with TNF for various periods of time in the absence (lanes 1–4, 6–9, and 11–14) or in the presence of ALLN (lanes 5, 10, and 15) (Fig. 6). Analysis of nuclear extracts by EMSA (A) showed a similar overall response of the cells isolated from the three categories of mice: we observed no modification of constitutive NF-κB DNA-binding activity nor of the kinetics of activation after TNF stimulation. ALLN treatment through inhibition of the proteasome prevented induction in a comparable fashion in all three genotypes. The use of specific Abs directed against individual members of the NF-κB family allowed us to define complex II as p50/p50 homodimers and complex I as p50/p65 heterodimers (data not shown). Analysis of cytoplasmic extracts by Western blotting (B) confirmed the absence of IκBε in T cells of homozygous mutant mice (bottom panel). No increase of p105 and IκBβ was detected in mutant thymocytes (upper panels). IκBβ was not degraded after TNF treatment in either wild-type or IκBε-deficient T cells. IκBα was up-regulated in resting mutant thymocytes and rapidly degraded and resynthesized after TNF treatment. Its degradation was inhibited by ALLN in both mutant and wild-type or heterozygous control cells. Stimulation of T cells with IL-1 again did not discriminate between mutant and control cells (data not shown).

Altered resting and Ag-specific Ab production in IκBε-deficient mice

To assess the humoral immunity of IκBε-null mice, we measured basal Ig levels of naive mutant mice or control wild-type littermates (Fig. 7A). IgG2a, IgG2b, and IgG3 levels are equivalent in both types of mice. A slight increase of IgM levels (2-fold) is detected in IκBε-deficient mice compared with control mice. The main difference between these mice lies in the augmentation (3-fold, with very small dispersion of the values obtained with the mutant mice, p < 0.001) of IgG1 seen in IκBε-deficient mice compared with wild-type mice.

We next analyzed the humoral response of IκBε-null mice or control wild-type littermates to specific Ab challenge. Mice were injected with LPS coupled to DNP to induce a T cell-independent response (B) or with KLH coupled to DNP precipitated in alum to induce a T cell-dependent response (C) and bled before and 7, 14,
and 21 days after immunization. DNP-specific Abs in unchallenged mice were very low and not significantly different in wild-type and mutant mice. For each Ag used, only DNP-specific IgG1 and IgM isotype productions were affected in IκBε-null mice; IgG2a, IgG2b, and IgG3 values were not statistically different from the wild-type values using the Student’s test.

Considering the T cell-independent response (B), DNP-specific IgG1 production in mutant mice increased by 3-fold at day 7, and this elevation persisted until day 21. DNP-specific IgM synthesis in the mutant mice was 2-fold higher at day 7 and 1.3-fold at day 14, and returned to levels similar to wild type by day 21.

For the T cell-dependent response (C), DNP-specific IgG1 secretion in IκBε-deficient mice differed from wild type only by a peak (1.5-fold increase) at day 7. DNP-specific IgM production rose by 2.5-fold at day 7 and remained elevated until day 21.

**Constitutive up-regulation of cytokines in IκBε-deficient mice**

Analysis of IκBε-associated proteins in cells, together with the finding that some NF-κB-responsive genes were activated solely by c-rel and/or p65 dimers, led to the hypothesis that the role of IκBε in vivo might be to regulate this specific subset of genes, which includes IL-8, tissue factor, and GM-CSF (15, 17). If this assumption true, these genes should have elevated basal expression levels in IκBε-deficient mice (43). If GM-CSF or the functional equivalent of IL-8 in the mouse was constitutively overexpressed in IκBε-deficient mice, one should expect, in a simplistic view, a quicker clearance of pathogenic bacteria. We thus asked how IκBε-null mice responded...
to challenge with a Gram-negative, *S. flexneri*, or with a Gram-positive, *L. monocytogenes*, bacteria. We used a previously described pulmonary model of *Shigella* infection by the nasal route, which mimics the lesions occurring in natural intestinal infection (31). As shown in Fig. 9A, high bacterial multiplication was observed in the lungs of wild-type mice 6 h after inoculation, the bacterial load decreasing after 24 h. An overall similar pattern of response to *S. flexneri* was obtained with heterozygote or homozygous *IκBε* mutant mice, the high SD between individual animals observed at 24 h ruling out any significant effect in the mutant mice. We next challenged the mice with *L. monocytogenes*. Results presented in Fig. 9B show that for every organ analyzed (liver, spleen, and brain), *IκBε*-null mice responded to the infection identically in intensity and kinetics to wild-type or heterozygote controls. This absence of bias between these three populations of mice persisted when we looked at the spleen 6 days after inoculation when bacterial loads were considerably diminished (data not shown). By using two distinct bacterial pathogens, one which led to localized bronchial epithelium invasion, and a second, which caused a systemic infection that ended in CNS invasion, we have been unable to establish any discrimination between the mutant and the wild-type control mice.

**Discussion**

The existence of three major *IκB* raises the question of their individual role and putative functional redundancy in vivo. The analysis of *IκBε*-deficient mice provides some clues about the function of *IκBε* in a whole organism. *IκBε*-null mice share none of the hallmarks of *IκBε*-deficient mice (23, 44): they survive to adulthood and show no increase in NF-κB DNA-binding activity in all organs and cell types analyzed (spleen, thymus, purified T cells, resident macrophages, or MEFs [data not shown]). They possess normal hemopoietic cell subsets, in particular displaying average granulocyte and macrophage numbers together with unaltered skin structure.

We have shown in this study that *IκBε* mRNA is expressed at high levels in the thymus, spleen, and to a lesser extent in lung and ovary, in accordance with observations in humans (16). Targeted replacement of the entire *IκBε* coding region by the lacZ gene carrying a nuclear localization sequence allowed us to visualize the overall expression pattern of *IκBε* in the immune system with...
A bacterial load was assessed. Results are expressed as the mean ± SD of CFU/gram of lung tissue from every inoculated mice. 7 to 23 wild-type (+/+), heterozygote (+/−), or IκBε-null (−/−) mice were intranasally infected with L. monocytogenes, lungs were removed after 6–24 h, and the bacterial load was assessed. Results are expressed as the means ± SD of CFU/gram of lung tissue from every inoculated mice. B, 5 to 7 wild-type (+/+), heterozygote (+/−), or IκBε-null (−/−) mice were i.v. infected with L. monocytogenes. Spleen, liver, and brain were removed after 1, 2, or 3 days (D-1, D-2, and D-3, respectively), and the bacterial load was assessed. Results are expressed as the mean ± SD of the log of CFU/organ from every inoculated mouse.

FIGURE 9. Normal responses of IκBε-deficient mice after pathogen challenge. A, 7 to 23 wild-type (+/+), heterozygote (+/−), or IκBε-null (−/−) mice were intranasally infected with S. flexneri, lungs were removed after 6–24 h, and the bacterial load was assessed. Results are expressed as the means ± SD of CFU/gram of lung tissue from every inoculated mice. B, 5 to 7 wild-type (+/+), heterozygote (+/−), or IκBε-null (−/−) mice were i.v. infected with L. monocytogenes. Spleen, liver, and brain were removed after 1, 2, or 3 days (D-1, D-2, and D-3, respectively), and the bacterial load was assessed. Results are expressed as the mean ± SD of the log of CFU/organ from every inoculated mouse.

cate that data from cell lines of the myeloid lineage, like THP1 or differentiated HL-60 cells, which display high endogenous levels of IκBε (15), cannot be extrapolated to primary cells. Considering the high sensitivity of FDG staining, which has been reported to detect as little as five molecules of β-galactosidase per cell (38, 45), and the fact that two independently generated knockin lines gave the same pattern of expression, it is quite unlikely that we overlooked any site of expression of IκBε in the lymphoid system.

Up-regulation of IκBα has been observed in several organs and cell types (i.e., thymus, spleen, purified T cells) isolated from IκBε-mutant mice. IκBβ was found overexpressed in the spleen, but not in purified T cells. These results suggest that the relative contributions of IκBα and IκBβ to the regulation of NF-κB are cell type dependent. The prominent role of IκBα is most likely due to its preferential expression in hemopoietic cells compared with IκBβ (44) and also to its autoregulation by NF-κB (46–50). IκBε expression is controlled by NF-κB, and IκBε protein levels are increased in primary IκBα-null MEFs (15). Up-regulation of other IκB species is therefore not a characteristic unique to IκBε-null mice. However, up-regulation of IκBα and IκBβ in the spleen of IκBε-nullizygous mice is intriguing since only 5% of FDG− cells were detected in this organ. It may suggest that overexpression of IκB family members could also take place in cells that do not normally express IκBε.

No significant increase in NF-κB-binding activity was detected in any IκBε-deficient organs examined or in purified T cells, of which 80% express IκBε. This suggests that cytoplasmic retention of NF-κB in IκBε-deficient mice is achieved by overexpression of IκBα and/or IκBβ. This functional compensation by IκBα and/or IκBβ for the lack of IκBε is most likely responsible for the discrete phenotype of IκBε-null mice. Nevertheless, substitution by these IκBαs is not complete and IκBε-deficient mice harbor a number of specific features, which might reflect specific functions of IκBε.

We observed a 50% decrease in the number of precursor CD44+CD25+ cells in spite of the presence of normal mature hemopoietic cell populations. Differentiation of thymocytes has been correlated with CD25 and CD44 expression during early steps of cortical maturation (51), and CD25 (or IL-2Rα-chain) expression in thymocytes is associated with TCR rearrangement. It is interesting to note that young mice lacking CD25 exhibit phenotypically normal T cell development (52). Lack of IκBε may impair expression of target genes involved in the proliferation of the CD44+CD25+ cell population and thus account for its diminution. Whereas CD25 gene expression was unaffected in IκBα-null mice (23, 44) and in homozygous mice deficient for the different members of the NF-κB/Rel family (53–59), a similar reduction in the frequency of CD25+ cells was detected in transgenic mice expressing constitutive trans-dominant form of IκBε (26). Reduction of the CD25+ cell population in IκBε-deficient mice might therefore result from an increase of IκBα, instead of being a direct consequence of IκBε loss. In this case, the up-regulation of IκBα should be assumed to be strictly limited to the CD25+ cell subset, since in IκBα transgenic mice this phenotype is associated with severe defects in T cell development and proliferation that are not observed in IκBε-null mice.

Another characteristic of IκBε-deficient mice is their increase in IgM and especially IgG1 basal levels. Recent reports have implicated NF-κB in the regulation of isotype switching. κB binding sites within the 3′ IgH enhancer (60, 61) and the germline CμH promoters, which regulate class switching to IgG1 and IgE (62, 63), were shown to bind specific NF-κB complexes, and analysis of the knockouts of the various NF-κB genes led to the observation that isotype switching was frequently affected in these mice. For instance, p50 mice displayed a significant reduction in all isotypes
except for IgM, which was slightly elevated (55). A pronounced reduction of IgG1 and IgA was seen in p65-deficient lymphocytes (56). RelB-deficient mice show increases in IgM, IgE, and IgG1 together with slight decreases in IgG2a, IgG2b, IgG3, and IgA (64). IgG1 and IgG2 were drastically reduced in c-Rel-null mice, IgM, IgG2b, and IgG3 being only slightly diminished (53). IgG1 and IgM levels appear to be frequently altered in NF-kB-deficient mice, and their increase in IxB-e-null mice might reflect modification of the balance of various NF-kB members in B cell subsets. Ab response to specific Ags was also modified in IxB-e-deficient mice. T cell-dependent and T cell-independent immunizations led to increased synthesis of IgM and IgG1. Impaired Ag-specific Ig isotype switching has also been observed in several NF-kB-deficient mice. C-Rel and RelB knockout mice exhibit defective T-dependent and T-independent Ab responses (53, 64), while an inability to generate Abs to T-dependent Ags has been observed in p52-null mice (58, 59). The increase in IgM and IgG1 levels before stimulation and after T-dependent or T-dependent challenge in IxB-e-nullizygous mice is, however, very modest compared with the perturbation observed for knockouts of the NF-kB gene family. Moreover, we have shown that only 1–2% of B cells express IxB-e. Alteration of isotype switching in IxB-e-deficient mice possibly reflects the contribution of these few IxB-e-expressing B cells. Alternatively, it is possible that this modification is indirectly due to IxB-e via the perturbation of other pathways such as cytokine synthesis.

IxB-e was proposed to be a specific regulator of genes controlled by p65 and/or c-Rel complexes, since it specifically interacts with IxB-e. Activation of isotype switching in IxB-e-deficient mice possibly reflects the contribution of these few IxB-e-expressing B cells. Alternatively, it is possible that this modification is indirectly due to IxB-e via the perturbation of other pathways such as cytokine synthesis.

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References


