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Innate Immune Responses in NF-κB-Repressing Factor-Deficient Mice

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NF-κB-repressing factor (NRF) is a transcriptional silencer protein that specifically counteracts the basal activity of several NF-κB-dependent promoters by direct binding to specific neighboring DNA sequences. In cell culture experiments, the reduction of NRF mRNA leads to a derepression of beta interferon, interleukin-8, and inducible nitric oxide synthase transcription. The X chromosome-located single-copy NRF gene is ubiquitously expressed and encodes a protein of 690 amino acids. The N-terminal part contains a nuclear localization signal, the DNA-binding domain, and the NF-κB-repressing domain, while the C-terminal part is responsible for double-stranded RNA binding and nucleolar localization. To study the function of NRF in a systemic context, transgenic mice lacking the NRF gene were created. Against predictions from in vitro experiments, mice with a deletion of the NRF gene are viable and have a phenotype that is indistinguishable from wild-type mice, even after challenge with different pathogens. The data hint towards an unexpected functional redundancy of NRF.

NF-κB/rel transcription factors regulate a variety of promoters through specific DNA-binding sites (12). Regulation is achieved mainly in the cytoplasm, where NF-κB is trapped in an inactive complex with the inhibitory molecule IκB (11). In addition, the activity of NF-κB is controlled at further levels. These include inducible phosphorylation (20, 22), binding of coactivators (25, 35), and repressors (17, 19, 33, 44).

NF-κB family members were detected in several cell types as a constitutively active nuclear complex (13, 20). Therefore, NF-κB/rel-binding sites often act as weak constitutive enhancers. This is one of the reasons why many NF-κB/rel promoters are constitutively active. On the other hand, a number of promoters which bear NF-κB/rel-binding sites are strictly regulated. They show no basal activity in the noninduced state. An example concerns mammalian beta interferon (IFN-β) gene expression. Apart from positive regulatory domains (PRDs) that are responsible for the inducible activation (7), a negative regulatory element (NRE) was identified and shown to be responsible for constitutive silencing. The NRE can act as a position-independent silencer of PRDII (28).

The so-far-characterized NREs represent a class of transcriptional repressor sequences with a silencing capacity targeted to the constitutive activity of NF-κB. NRE-related sequences were found and verified in the promoters of human immunodeficiency virus type 1, human T-cell leukemia virus type 2 (29), and those of IFN-β, interleukin-8 (IL-8), and the inducible nitric oxide synthase (iNOS) gene (8, 31). The common features exerted by the presently investigated five NREs are sequence homology, short length (11 to 13 bp), distance- and position-independent action, specific silencing of NF-κB/Rel-binding sequences, and indistinguishable binding patterns to nuclear factors.

Human cDNAs of a protein that bind to NRE sequences were identified (27, 30). Their analysis revealed a single-copy NRF gene that is located on the X chromosome. The sequence of the encoded protein has 690 amino acids and was denominated NRF (NF-κB-repressing factor). NRF mRNAs are constitutively expressed in all tested human adult tissues. While NRF mRNA is transcribed in embryonic stem (ES) cells, its expression in embryonic development is not homogenous (39).

The involvement of NRF in the promoter regulation of IFN-β, IL-8, and iNOS was studied by NRF-antisense mRNA experiments. Induction of endogenous IFN-β, IL-8, and iNOS was found at low but significant levels as a consequence of mRNA ablation (8, 30, 32).

The NRF protein contains several functional domains. Within the first 380 amino acids the DNA (NRE)-binding domain and a domain responsible for NF-κB repression were identified (30). The C-terminal part contains several sequence motifs that are responsible for NRF binding to double-stranded RNA (dsRNA) and for its localization to the nucleolus. Due to an N-terminal nuclear localization sequence, NRF shows a constitutive nuclear accumulation. In vitro, NF-κB proteins bind to purified NRF by a direct protein-protein interaction, and in a cellular assay NRF inhibits NF-κB basal activity (30).

In this report we describe the creation and properties of transgenic mice in which the NRF gene was deleted through homologous recombination in ES cells. A knockout strategy

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was applied in which the C-terminal part of the protein is constitutively deleted and the N-terminal part is conditionally eliminated. According to the data revealed from NRF activity in cell culture experiments (8, 39), the expectation was that these mice would show dysregulated innate immune responses. In contrast to this expectation the mice are viable, reproductive, and show no obvious phenotype even after challenge with pathogens.

MATERIALS AND METHODS

Protein and nucleic acid sequence databank searches. The following DNA and protein sequence analysis programs and databases were used: blastp, tblastn, and CD-Search (24) and pfam searches (the Pfam Protein Families Database [2]).

Generation of NRF transgenic mice. The NRF targeting vector (see Fig. 2A, below) carries the chromosomal NRF gene, in which 5′ half of the second exon is flanked by two loxP sites, with insertion of an artificial STOP codon at the 3′ loxP site. The neomycin resistance gene owns a truncated tk promoter. DNA was electroporated into E14-1 ES cells derived from the 129/Sv mouse and cultured on a layer of mitotically inactive feeder cells. ES cell colonies resistant to neomycin were expanded and screened for homologous recombination by PCR and Southern blot analysis of SpeI digests by using internal (second exon) and external (5′-end) probes. One selected ES cell clone exhibiting a normal karyotype was injected into C57BL/6 blastocysts to obtain chimeric animals (Mice & More, Hamburg, Germany). Chimeric male mice with a high contribution of agouti coat color were bred to C57BL/6 females, and germ line transmission of the NRF flox allele was obtained. Genotypes of these mice were confirmed by Southern blotting of tail DNA. Subsequent genotyping was carried out by PCR with allele-specific primers (data not shown; experimental details are available upon request). The PCR primers used were as follows: 5′-itr, 5′-GTC TCT GGT ATA GCC TTA GTA GTG GG-3′, or 2nd exon, 5′-GTT CTG CCA AAC ACT GGA CC-3′ (forward); 3′-end, 5′-CTG AGA TAG GCT CCC GTA TGC CC-3′ (reverse); Neom, 5′-CCG CTT CCT CGT GCT TTA CGG-3′.

Mice. To obtain specific-pathogen-free animals, mice of the F_1 generation were rederivated by embryo transfer. NRF transgenic mice were then bred and kept under standard pathogen-free conditions in the animal facility of the German Research Center for Biotechnology (Braunschweig, Germany). Mice were used at 12 to 16 weeks of age.

To get mice carrying a functional Mx1 gene, NRF-KO females were bred with $Mx1^{+/+}$ males. Male offspring are heterozygous at the Mx1 locus and have a defective NRF gene. Female offspring resulting from this breeding are heterozygous at the Mx1 locus and carry one functional NRF allele. Mx1 breeding and virus challenge experiments were carried out at the animal facility of the University of Freiburg, Freiburg, Germany.

Isolation and culture of primary cells. Primary dermal fibroblasts were isolated from 2-day-old mice. Trunk skin was removed, washed, and incubated overnight at 4°C in 0.25% trypsin (Gibco). The dermis layers were then mechanically separated from the epidermis, treated with 0.15% collagenase A for 30 min at 37°C, and then mechanically dissociated. The cell suspension was centrifuged for 5 min at 1,200 rpm. The cellular pellet was suspended and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Peritoneal macrophages were obtained by peritoneal lavage of sacrificed mice with cold phosphate-buffered saline (PBS). The cells from two to five mice were combined, centrifuged at 1,200 rpm, resuspended in medium, and plated. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum.

Detection of IFN-α/β and NO production. The IFN-β concentrations in supernatants from induced and control cells were determined by an antiviral assay using mouse LMTK cells infected by vesicular stomatitis virus (VSV) as described elsewhere (6).

The accumulation of NO_2, a stable product of NO metabolism, was measured as an indicator of NO production using Griess reagent [1% sulfanylamine in 30% acetic acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 60% acetic acid; 1:1, vol/vol; Sigma]. The absorbance at 540 nm was measured in a microplate reader. The NO_2 concentration was calculated from a standard curve of sodium nitrite.

Expression analysis by RT-PCR. Preparation of total RNA (RNeasy kit; QIAGEN) and first-strand cDNA synthesis (Superscript II; Gibco) were done according to the manufacturers' instructions. Reverse transcription-PCR (RT-PCR) analysis of IFN-β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression were performed with following primers: IFNB_sense, 5′-CAT CAA CTA TAA GCA GCT CCA-3′; IFNB_antisense, 5′-TTC AAG TGG AGA GCA GTT GAG-3′; GAPDH_sense, 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH_antisense, 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Infection with *Listeria monocytogenes* and lipopolysaccharide (LPS) challenge. *L. monocytogenes* EGD (serotype 1/2a) stock cultures were maintained at −70°C and cultured overnight on a brain heart infusion (BHI; Difco) agar plate at 37°C. A single colony was inoculated in 5 ml BHI broth and incubated overnight at 37°C. The bacteria culture was diluted 1:10 in BHI and cultured until mid-log phase. Bacteria were washed with PBS, viable bacteria were counted using a Thoma chamber, and counting was confirmed by plating serial dilutions. Mice were injected with 1.5×10^4 CFU of *L. monocytogenes* via a lateral tail vein.

For challenge with LPS, mice were injected intraperitoneally with 250 μg/mouse of LPS (*Salmonella enteritidis* serovar Abortus Equi) freshly prepared in PBS.

The condition of the mice was determined by daily measurement of animal body weight and observation of mouse behavior. No difference was found between wild-type (WT) and NRF knockout (NRF-KO) mice.

Generation of NRF-specific antibodies. A 21-amino-acid peptide was synthesized with the amino acid sequence C(-SH)-DQLKQEGQVGHYELVMPQAN-(COOH) that was derived from the putative C-terminal sequence of the NRF protein. Five milligrams of the high-performance liquid chromatography-purified peptide was coupled to *Limulus polyphemus* hemocyanin as a carrier (Biogenes, Berlin, Germany). Two rabbits were immunized on day 1, and 1.5 ml of preimmune serum was withdrawn. Subsequent immunizations were on day 7, day 14, and day 28. Twenty milliliters of blood was drawn at day 35, and a final bleeding at day 42 yielded 60 ml serum. Antibodies were subsequently affinity purified using the same peptide (Biogenes, Berlin, Germany).

To confirm the specificity of the NRF antibody, nuclear extracts isolated from C243 cells that overexpress a myc-tagged NRF protein were analyzed by consecutive blotting with antibodies against NRF and the myc tag. Both antibodies recognize the same protein band (data not shown).

Western blotting, immunofluorescence, and fluorescence-activated cell sorter analysis. Nuclear protein extracts were prepared using the NucBuster protein extraction kit (Novagen) as described in the manufacturer's instructions. Western blot analysis was performed using antibody directed against the NRF protein followed by secondary horseradish peroxidase-conjugated anti-rabbit antibody and the enhanced chemiluminescence detection method (Pierce). Immunofluorescence analysis was performed as described previously (27). Anti-NRF antibody was used at a 1:250 dilution followed by Cy3-labeled anti-rabbit antibody (Dianova) at a 1:800 dilution. Lymphocytes obtained from the peripheral blood were analyzed as described earlier (9, 21, 38).

FISH. Chromosomes were analyzed by fluorescence in situ hybridization (FISH) (23) using an NRF gene-specific BAC clone. The BAC clone was labeled by nick translation using digoxigenin-11-dUTP-conjugated nucleotides (Roche). For visualization of the X chromosome, labeled DNA from a murine X chromosome library was used. Chromosomes were prepared from a normal male mouse ES cell line (ES 14.1) using a standard protocol (43).

Histology. Organs for histology were harvested and fixed in 10% neutral-buffered formalin, dehydrated through a graded series of alcohol, embedded in paraffin, sagittally sectioned at 5-μm intervals, and processed for hematoxylin and eosin staining according to standard protocols.

DNA microarray hybridization and data analysis. Total RNA was isolated from WT and NRF-KO primary mouse fibroblasts using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Quality and integrity of the total RNA was confirmed using the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). Biotin-labeled target synthesis was performed using standard protocols supplied by the manufacturer (Affymetrix; Santa Clara, CA). Briefly, 5 μg total RNA was converted to dsDNA using 100 pmol of a T7T23V primer (Eurogentec, Seraing, Belgium) that contains a T7 promoter. The cDNA was then subjected to in vitro transcription in the presence of biotinylated nucleotides to generate biotin-labeled cRNA. All samples were hybridized to the same lot of Affymetrix MOE430A for 16 h. After hybridization the GeneChips were washed, stained with streptavidin-phycoerythrin, and read using an Affymetrix GeneChip fluidic station and scanner.

Analysis of microarray data was performed using the Affymetrix Microarray suite 5.0, Affymetrix MicroDB 3.0, and Affymetrix Data Mining Tool 3.0. For normalization, all array experiments were scaled to a target intensity of 150, otherwise using the default values of the Microarray suite. Filtering of the results was done as follows: genes were considered regulated when their change was greater than or equal to 2.5-fold or less than or equal to –2.5-fold (change "A"); the statistical parameter for a significant change was less than 0.01 (change in *P* value for changes called increased [I]) or greater than 0.99 (change *P* value for changes called decreased).

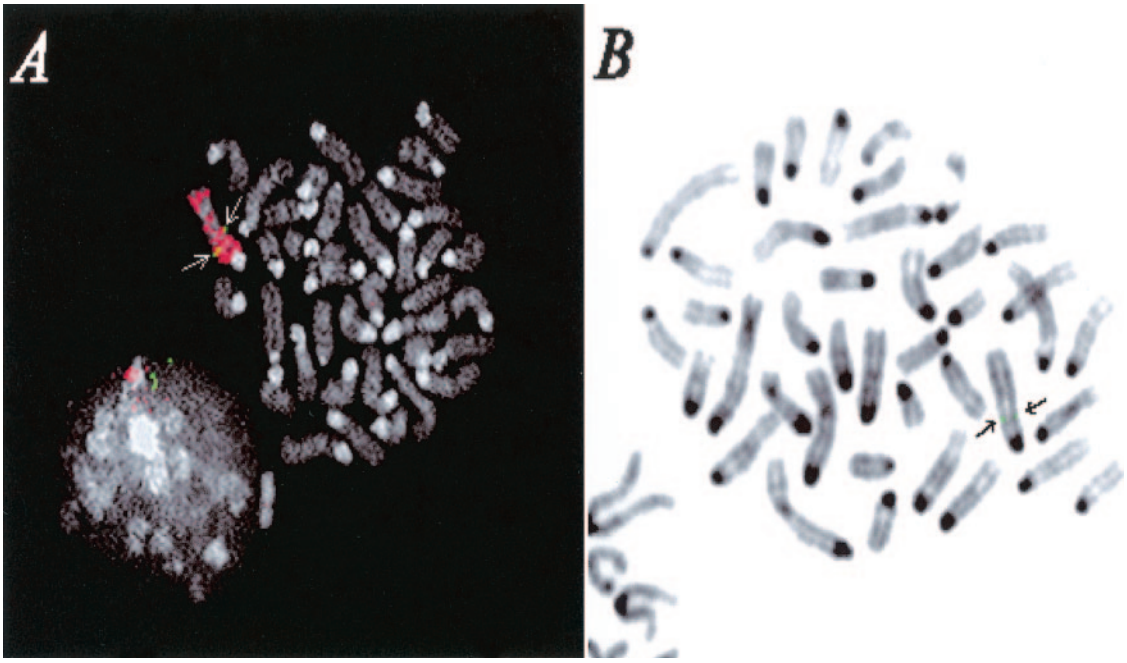

FIG. 1. Chromosomal localization of murine NRF by FISH analysis. The ES cell line 14-1 was hybridized with BAC DNA harboring the NRF gene. The NRF gene is labeled in green and marked by arrows. A. X chromosomal DNA is counterstained in red. B. Chromosomes of a different spread were counterstained with 4′,6′-diamidino-2-phenylindole.

RESULTS

Identification of the murine NRF sequence. Previous reports about function and structure of NRF have made use of the human cDNA sequence (30). To study NRF function in mice, a murine cDNA was isolated by hybridization of an 11.5-day embryo mouse cDNA library (Clontech) with the human NRF sequence. The nucleotide sequence of the murine NRF shows 93% homology to the human counterpart. The comparison of the amino acid sequences revealed that the C-terminal part (302 amino acids) of the NRF protein is strongly conserved (297 amino acids), whereas the N terminus shows some divergences (28 amino acids in 388). In situ hybridization analysis indicates that the murine NRF gene is located on the X chromosome, region A4 (Fig. 1). This is also the case for the human homolog, as it could be found in public DNA databases. In silico analysis identified evolutionarily conserved domains within the C-terminal part of the NRF protein that are abundant among nucleic acid binding proteins (27). However, we found no evidence for a second NRF-encoding gene, nor did we identify another gene with a relevant overall similarity to the NRF protein in the murine genome. Thus, the NRF genes from mouse and human are unique with no close relatives.

Generation of NRF knockout mice. To study effects of NRF deletion, transgenic mice expressing mutant forms of the NRF protein were created. Using the murine cDNA as a probe, a BAC clone from a chromosomal library derived from 129 mice was isolated and analyzed (data not shown). A targeting vector was constructed (39). The knockout strategy is outlined in Fig. 2. Mice carrying the transgenic cassette were denominated "NRF-flox." In these mice, a short form of NRF is encoded (NRF-flox). This form was shown to carry an NLS, the DNA-binding domain, and the NF-κB-interacting domain (30). Upon breeding of the NRF-flox mice to the K14Cre deleter strain (14), expression of Cre-recombinase leads to excision of the second exon and results in the deletion of the NRF protein coding sequence. These animals were designated "NRF-KO" (Fig. 2A).

The transgenic genotypes of NRF-flox and NRF-KO mice were confirmed by Southern blotting. (Fig. 2B). Transcription from the NRF-KO allele is still possible because of the presence of the 5′ region and the first exon that encodes 37 amino acids. Since the second exon is deleted together with a fragment of the first intron, including the splice acceptor, mRNA from the NRF-KO cells is not expected to be processed by splicing. Thus, the predicted mRNA from NRF-KO cells contains the 5′ part of the intron sequence. Multiple STOP codons present in this intron region prevent translation of the unspliced mRNA. A potential translation product would have 45 amino acids. The NRF-flox-derived mRNA contains sequences encoding the N-terminal half of the NRF protein and the neomycin phosphotransferase. Accordingly, its size is 5.2 kb (Fig. 2C). Northern blot analysis of RNA from WT, NRF-flox, and NRF-KO cells is shown in Fig. 2D. No sign of NRF mRNA in the samples isolated from NRF-KO cells was detected, probably because of the instability of intron sequence-containing RNA. Thus, NRF-KO cells produce, if at all, a 45-amino-acid N-terminal peptide of NRF. This led us to conclude that no functional NRF protein is produced in NRF-KO mice.

NRF-KO mice were born at Mendelian ratios expected for a single X chromosome-linked gene. Mutant NRF homozygous and heterozygous mice show no difference in size, behavior, and reproductive ability compared with WT littermates. Ho-

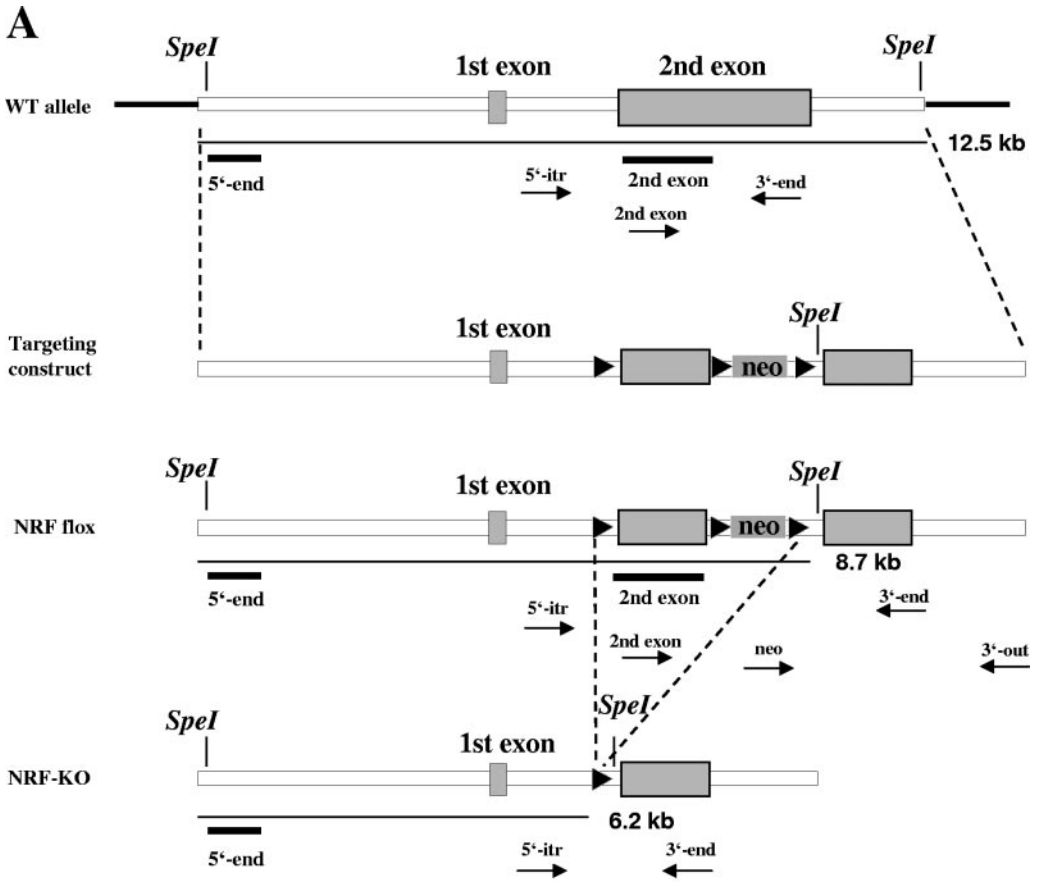

FIG. 2. Targeted disruption of the mouse NRF gene. A. Schematic structures of the murine NRF gene, the targeting vector, and the genomic modifications in transgenic mice are shown. Homologous DNA is boxed. The orientation and locations of primers (arrows) and a probe fragment for genotyping (bars) are indicated. B. Southern blot analysis of DNA from transgenic mice. Genomic DNA isolated from tail biopsy samples was digested with SpeI and hybridized with the second exon (left panel) and 5′-end (right panel) specific probes as indicated. hom, homozygous; het, heterozygous mice. C. Schematic structure of WT, NRF-flox, and NRF-KO DNA forms and the expected mRNA. Note that RNA from NRF-KO is not detectable. D. Northern blot analysis. Poly(A)-RNA was isolated from WT, NRF-KO, and NRF-flox cells. The RNA blot was hybridized with a radioactively labeled NRF second exon-specific probe. For each sample, 2 μg RNA was loaded.

mozygous NRF-KO mice thus revealed that the lack of the NRF gene is compatible with life and does not cause any visible pathological effect.

Localization of murine NRF protein. To determine NRF expression, nuclear extracts from WT and NRF-KO primary fibroblasts were subjected to Western blot analysis using specific antibodies directed against the C-terminal end of NRF (Fig. 3A). The calculated molecular mass of the murine NRF protein is 77.6 kDa. In nuclear lysates isolated from WT cells, a specific band around 80 kDa can be detected. Accordingly, in lysates isolated from KO cells no NRF-specific protein was found.

Indirect immunofluorescence was used to determine subcellular localization of the NRF protein in primary fibroblasts isolated from WT and NRF-KO animals. Localization of the endogenous NRF protein is restricted to nucleoli (Fig. 3B). This confirms the nucleolar localization of a human recombinant NRF protein (27). Thus, our data obtained for the endogenous protein in murine cells are in agreement with a general nucleolar localization of NRF. In NRF-KO cells no specific staining with anti-NRF antibodies was detected, confirming the absence of the NRF protein.

Histological analysis of NRF-KO mice. Constitutive activation of the NF-κB pathway is associated with chronic inflammatory diseases, and its dysregulation leads to proliferation and differentiation disorders in the skin (3). Since NRF acts as a constitutive silencer of certain NF-κB-regulated promoters, similar effects could be expected after elimination of the NRF gene. Therefore, a histological analysis of tissue sections from the organs of NRF-KO mice was carried out. No obvious differences in organ morphology were detected compared to WT animals. For illustration, representative sections of skin, joints, and lymph nodes from WT and NRF-KO mice are shown in Fig. 4.

Analysis of peripheral blood cells. NF-κB-driven genes play crucial roles in lymphocyte proliferation, differentiation, and apoptosis and thus in the composition of the hematopoietic cell population (1, 10). Since some of the promoters of the NF-κB-driven genes harbor NREs, deletion of a functional NRF could lead to an alteration in the hematopoietic cell

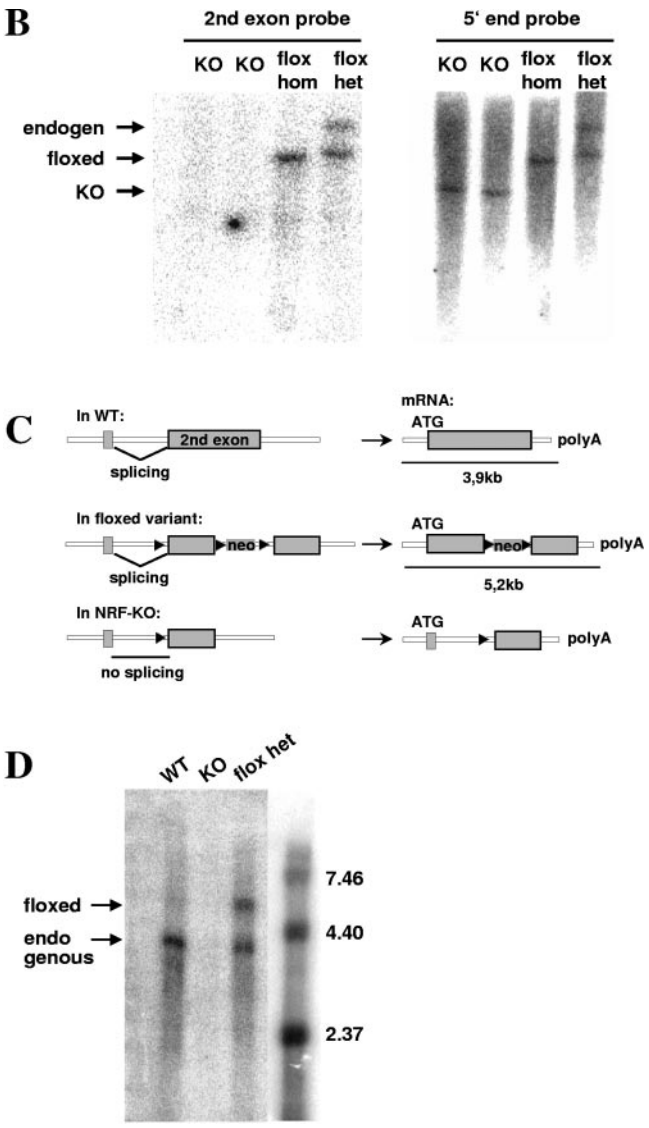

FIG. 2—*Continued.*

population. Peripheral blood mononuclear cells from WT, NRF-flox, and NRF-KO mice were analyzed by flow cytometry. Mature and immature B cells, CD4 and CD8 T cells, NK cells, neutrophils, and monocytes/macrophages were distinguished. No significant differences were found in the percentage of these peripheral blood cell subpopulations when NRF-flox and NRF-KO mice were compared to wild-type mice (Fig. 5).

IFN-β expression in NFR-KO cells. In vitro studies showed that NRF has a constitutive silencing activity on the IFN-β promoter (30). We investigated effects of NRF deletion on the murine IFN-β gene expression in a homogenous population of primary fibroblasts from skin of newborn mice. To determine significant production of IFN in the absence of virus, fibroblasts derived from WT, NRF-flox, and NRF-KO mice were subjected to an antiviral assay (see Materials and Methods). NRF-KO fibroblasts do not constitutively secrete IFN-β. In addition, total RNA isolated from WT and NRF-KO cells was analyzed by RT-PCR (Fig. 6B). With this more sensitive method, no sign of IFN-β RNA was found in nonstimulated cells, confirming that neither WT nor NRF-KO cells produce this cytokine constitutively. To test if these primary fibroblasts are able to produce equal amounts of IFN-β upon activation, cells were infected with Newcastle disease virus and IFN-β secretion was measured. Similar amounts of IFN-β were detected in all tested cell lines upon virus stimulation, indicating that activation of the IFN-β promoter was not altered by NRF deletion (Fig. 6A). IFN-α/β antagonize efficiently viral replication in cultured cells. To evaluate this response in NRF-deficient fibroblasts, cells from WT and NRF-KO mice were infected with VSV and viral replication was determined by a titration assay. Compared to WT cells, VSV replication was not inhibited in the absence of NRF (data not shown), which is consistent with the finding that no differences exist between NRF-KO and WT cells in this respect.

iNOS production by NRF-KO cells. Because of the transcriptional repression of the iNOS gene by NRF (8), it was hypothesized that a lack of the NRF inhibitory action on the iNOS promoter could cause constitutive expression of this enzyme and lead to accumulation of NO under noninduced conditions. Macrophages are known to express high levels of iNOS. Peritoneal macrophages were isolated from nonstimulated WT and NRF-KO mice, and iNOS activity was analyzed. Figure 6C shows the rate of the NO metabolite NO_2 determined in WT and NRF-KO cells after induction with LPS. From these data we conclude that regulation of the iNOS gene in primary peritoneal macrophages is not affected by NRF deletion. To confirm the absence of iNOS expression in NRF-KO cells, mRNA from untreated macrophages was subjected to RT-PCR analysis. NRF deletion failed to induce detectable amounts of iNOS mRNA (data not shown).

Gene expression profiling of the NRF-KO fibroblasts. In order to determine the genes whose expression is affected by the NRF deletion, we applied microarray analysis, which allows large-scale profiling of gene expression. For this analysis, RNA was isolated from homogenous populations of primary fibroblasts from skin of newborn WT and NRF-KO mice. WT cells were isolated from the female mice, whereas NRF-KO cells were derived from the male animals. A difference in the expression level of sex chromosome-specific genes (inactive X-specific transcript, eukaryotic translation initiation factor 2, and DEAD box polypeptide) served as an internal control of the method. From 24,000 probe sets corresponding to more than 15,000 genes, only 12 genes were found to be differentially expressed (more than 2.5-fold) in NRF-KO cells compared to WT cells (Table 1). A relation to a defined phenotype was not possible.

Systemic effects of the NRF deletion. Infections challenge the immune system and perturb many physiological parameters. NRF-KO mice were challenged with different bacterial and viral pathogens. The ablation of NRF function could either lead to higher resistance towards infection or to defects in the mechanisms of host defense. Since the type of the immune response depends on the nature of the pathogen and infections can induce a broad range of intracellular pathways, NRF-deficient mice were infected with different bacterial and viral pathogens.

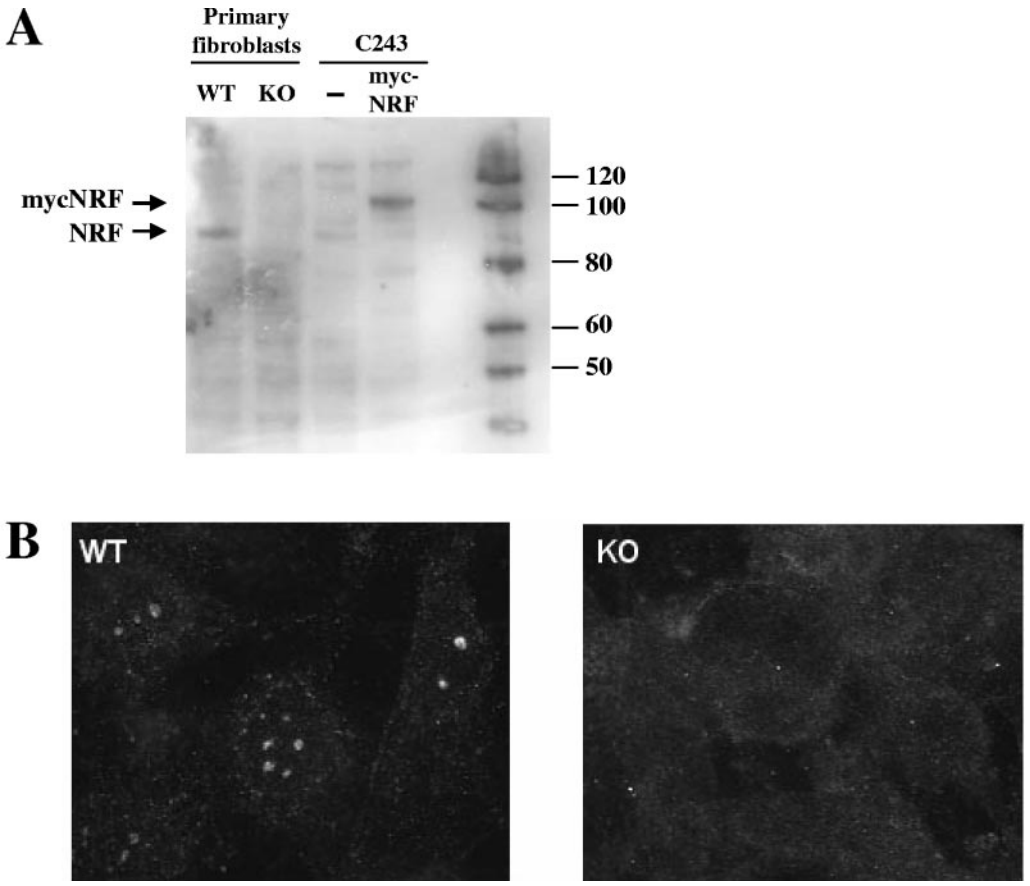

FIG. 3. Expression analysis of the NRF protein. A. Western blot analysis. Nuclear extracts were prepared from WT and NRF-KO primary cells as well as from the mouse C243 cell line either empty (-) or overexpressing the myc-tagged NRF protein. For primary cells 30 μg was loaded per lane, and for C243 cells 15 μg of protein was loaded per lane. The protein blot was incubated with anti-NRF antibody and treated as described in Materials and Methods. B. Immunofluorescence of the WT and NRF-KO cells using antibody directed against NRF. The secondary antibody was fluorescence labeled. Visualization was performed by confocal laser scanning microscopy (see Materials and Methods).

Susceptibility to LPS-induced shock. Bacterial LPS is the primary pathogenic factor in gram-negative bacteria. To address the question whether NRF deletion would affect the susceptibility to LPS, NRF-KO animals and their heterozygous littermates were injected with a high dose of LPS (250 μg/mouse) and the time of death of individual mice was monitored. We could not detect any difference in the survival rate after LPS injection. All mice (NRF-KO and their heterozygous littermates) developed severe symptoms of the LPS-induced shock and died within 48 h, indicating that the NRF deletion could not rescue animals from the lethal effects of LPS.

Susceptibility to infection with *L. monocytogenes*. *Listeria monocytogenes* has been widely used as a model to investigate the pathogenesis of intracellular bacterial pathogens and the regulation of cellular immunity (4). To address a potential function of NRF in fighting bacterial infection, NRF-KO mice and their heterozygous littermates were subjected to an intravenous injection of 1.5×10^4 CFU of *L. monocytogenes* and their survival rate was monitored. No obvious differences in survival rate of female WT and NRF-deleted mice were observed (Fig. 7). Male mice are more resistant towards infection with *L. monocytogenes* (34). Therefore, WT and NRF-KO males that survived infection for more than 2 weeks were subjected to histopathological examination. As expected for animals that had overcome infection, lymphocyte infiltration into various organs was observed (data not shown). However, no difference between WT and NRF-KO mice could be found.

Susceptibility to viral infection. Influenza virus, a member of *Orthomyxoviridae*, causes high mortality and represents a frequently used model for viral infection studies. NRF-KO mice were intranasally infected with 10^4 PFU of a mouse-adapted variant (844) of influenza A virus strain PR8 (H1N1) by intranasal application. Similar to WT mice, NRF-KO animals did not survive this infection, indicating that the NRF deletion has no detectable effect on influenza A virus resistance.

Borna disease virus (BDV) infection can result, depending on host and age, in symptomless viral persistence or severe immune-mediated neurological disease (5, 41). BDV induces only very low levels of IFN in brains of infected mice (42). On the other hand, it is known that BDV is highly susceptible to IFN (42), indicating that the virus would be blocked efficiently if IFN were present. If NRF-KO mice produced IFN more readily in response to virus infection, growth of BDV should be inhibited in the brains of these animals. To evaluate this possibility, 13-day-old animals were injected intracerebrally with 100 PFU of a mouse-adapted derivative of BDV strain RW98 (18).

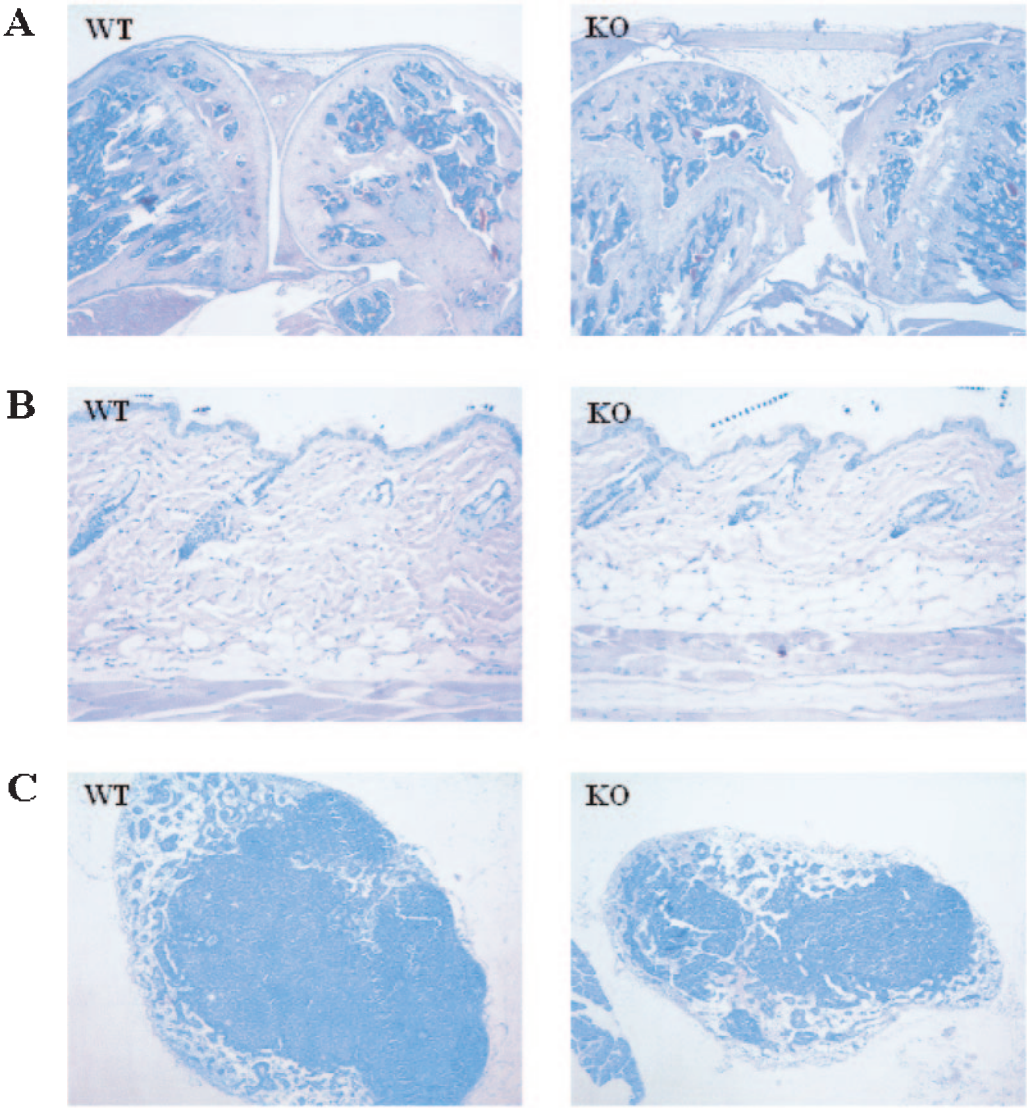

FIG. 4. Histological analysis of tissue sections from WT and NRF-KO mice stained with hematoxylin and eosin. (A) Joint; (B) skin; (C) lymph nodes.

Five of seven infected NRF-KO animals developed strong neurological disease between days 23 and 29 postinfection. Analysis by immunohistochemistry indicated that these animals contained large amounts of BDV antigen in the brain like infected wild-type mice, indicating that BDV did not induce enhanced levels of IFN in the brain of mice that lack NRF.

Thogoto virus (THOV) is the prototype tick-transmitted orthomyxovirus (37). Standard laboratory mice that lack a functional Mx system are highly susceptible to THOV. By contrast, mice that carry a functional Mx1 gene derived from wild strains are highly resistant if infected as adults but susceptible if infected as newborns (36). It was recently shown that even small amounts of IFN can efficiently activate the Mx system and protect newborn Mx1-positive mice from the lethal effects of THOV (36). Newborn NRF-KO males and their heterozygous female littermates carrying a functional Mx1 gene were injected with THOV. If the absence of a functional NRF gene would cause enhanced IFN synthesis, newborn

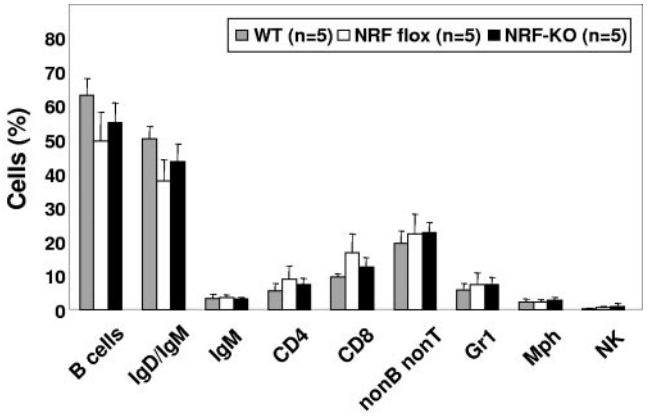

FIG. 5. Composition of the peripheral blood cells. Lymphocytes obtained from WT, NRF-flox, and NRF-KO mice were analyzed by fluorescence-activated cell sorter. The percentage of the indicated population is indicated.

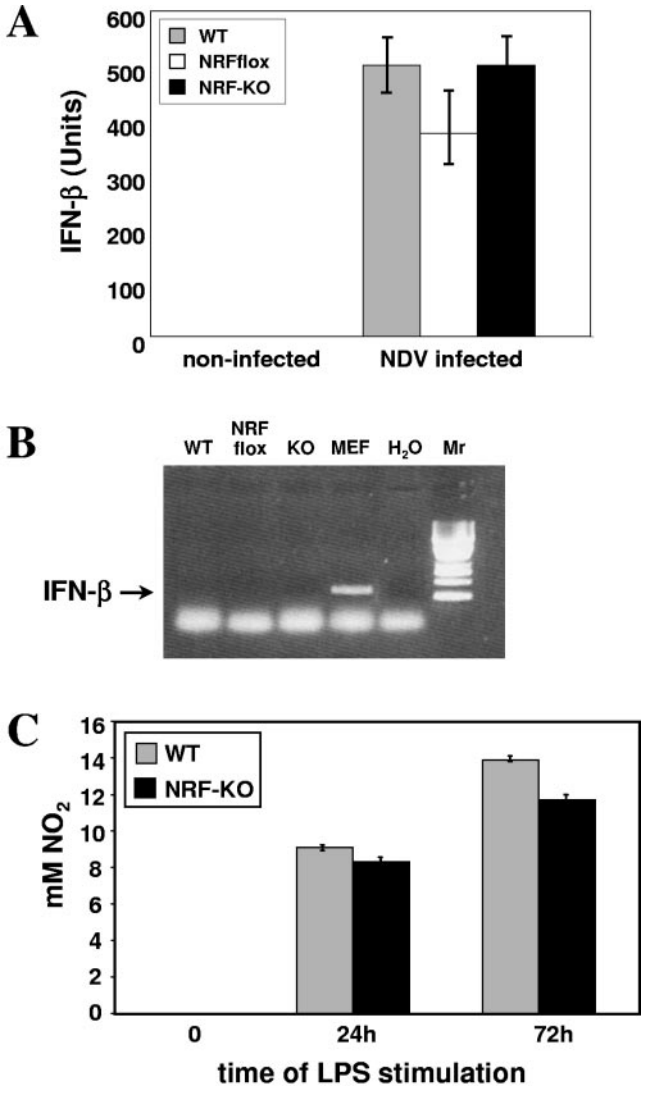

FIG. 6. Expression of IFN-β and iNOS. A. IFN-β production was determined in nontreated and Newcastle disease virus (NDV)-injected WT, NRF-flox, and NRF-KO primary fibroblasts as indicated. B. RNA isolated from cultured adult skin fibroblasts from WT, NRF-flox, and NRF-KO mice or WT mouse embryonic fibroblasts (MEFs) was isolated. mRNA was reverse transcribed into cDNA and subjected to PCR analysis using IFN-β-specific primers. As a positive control, RNA isolated from MEFs was used. These cells are known for their ability to produce a low level of IFN-β in the nonstimulated state (R. Zawatzky, personal communication). C. iNOS activity in peritoneal macrophages. Freshly isolated macrophages from WT and NRF-KO animals were stimulated with 10 mg/ml LPS. NO_2 was measured in the supernatants 24 and 72 h after LPS stimulation using Griess reagent.

males would be more resistant to THOV than females. We found, however, that all infected pups ($n = 13$) of both sexes died within 7 days, indicating that the NRF deletion did not influence IFN production in THOV-infected mice.

DISCUSSION

The knockout of the murine NRF gene was undertaken to study the biological implications of NRF in a systemic context. All data published so far concern experiments carried out in tissue culture with immortalized cell lines. The results gained from these studies have pointed towards an important function of NRF in the fine regulation of the NF-κB action on several promoters. The knockout of the gene was thus expected to have significant implications in a whole animal system. However, it is well known that cell culture experiments can be misleading, because many of their properties are significantly changed by the immortalization process and the long time of cultivation ex vivo. In vitro cultivation does not reflect the situation of a cell in natural surroundings, since its interplay with other cells and cell types in a three-dimensional setting with numerous regulatory loops is missing. The regulation of NF-κB promoters is such an example. Many cell lines show a high level of expression of members of the NF-κB/rel family (e.g., HeLa cells). Feedback regulations that involve extracellular factors or cell-cell signaling are often missing in cell culture.

The innate immune system is highly evolved, and it has to be expected that redundancy with respect to single essential molecular functions has been developed over millions of years.

TABLE 1. Examples of differentially expressed genes

Gene	Fold change[a]
DEAD box polypeptide, Y chromosome	344.89
Eukaryotic translation initiation factor 2, structural gene, Y linked	11.47
Growth arrest specific 5	10.41
Adenylyl cyclase-associated CAP protein homolog 1	4.03
P lysozyme structural	3.63
RIKEN cDNA 2610042L04 gene	3.20
ATPase, H^+ transporting, lysosomal (vacuolar proton pump), subunit 1	−2.50
Myosin light chain, alkali, cardiac atria	−2.55
Rous sarcoma oncogene	−3.18
Matrix metalloproteinase 3	−3.46
Schlafen 2	−7.57
Inactive X specific transcript	−91.77

[a] Positive values represent genes for which expression was up-regulated, and negative values indicate those with down-regulation in NRF-KO samples.

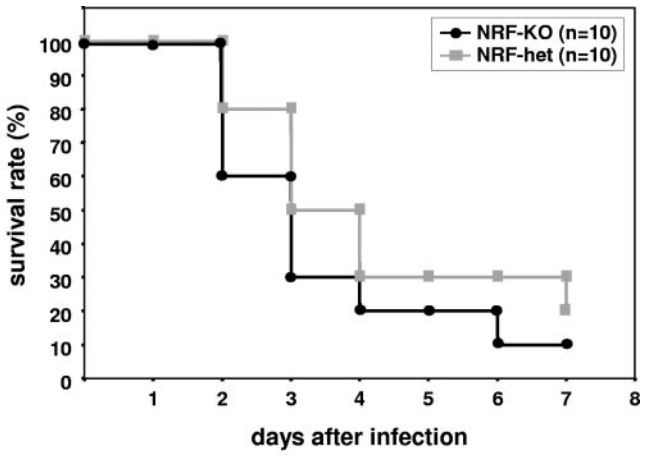

FIG. 7. Survival rate of NRF-KO female mice and their heterozygous littermates after injection with 1.5×10^4 CFU of *L. monocytogenes*. The condition of the mice was determined by daily measurement of animal body weight and observation of mouse behavior.

This could also apply to the regulation of the innate mediators IFN-β, iNOS, and IL-8, which were shown to be regulated by the NRF function. For example, in the regulation of the IFN-β promoter, several mechanisms prevent its activity in the absence of specific signals: (i) NF-κB signaling alone is not sufficient to induce the IFN-β promoter; synergism of IRF-3 and NF-κB is needed (40). (ii) The position of nucleosomes blocks the activity of the promoter in the noninduced state, and only a timely correct assembly of the inducing factors allows its removal (26). (iii) The NRF activity blocks the NF-κB activity in the absence of induction (30).

The results presented in this report show that the deletion of the NRF gene does not affect essential functions of mice. They also show that the expected derepression of the genes encoding IFN-β and iNOS does not take place in the examined cells of the adult mice. It cannot be excluded that this is the case for other cell types. However, a systemic effect from such activities would be expected and should affect the composition of peripheral blood lymphocyte, the infection by some viruses, and most likely also the histology of the examined tissues.

We have also challenged the KO mice with diverse pathogens in order to perturb a multitude of functions and to uncover a possible phenotype. Because the animals were not genetically homogenous, we took animals from the same litters to compare KO and WT NRF genotypes. Again, it is impossible to exclude small differences in these assays. However, a significant effect would have been detected.

Since none of these reactions was seen, we conclude that the examined genes were not derepressed. The consequent conclusion is that the effect of NRF is compensated by an unknown mechanism. We have searched public nucleic acid sequence databases for open reading frames that could encode NRF-related proteins. Potential NRF protein ortholog sequences were identified in various mammals, birds, and fish. Proteins with a domain structure related to the NRF N and C terminus were even detected in insects (*Drosophila melanogaster* gi21357453). Another evolutionarily conserved mammalian protein, CARF (Unigene HS32922), shows sequence similarity to a fragment of NRF (amino acids 167 to 318) (15, 16). However, CARF lacks sequences homologous to the C-terminal NRF sequences, including the proposed DNA-binding domain, and may therefore not replace the proposed sequence-specific repression function of NRF (30). We thus assume that it is not a related protein that fulfills the molecular function of NRF. It is possible that another protein, e.g., a DNA-binding protein that recognizes the same DNA sequence, overtakes its function. Alternatively, an unrelated mechanism may exist that leads to the observed compensation.

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