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Article **Immunology**

IκB proteins play an important role in regulating NF-κB induction following a diverse range of environmental injuries. Studies evaluating IκBβ knock-in mice (AKBI), in which the *IκBα* gene is replaced by the *IκBβ* cDNA, have uncovered divergent properties of IκBα and IκBβ that influence their ability to activate hepatic NF-κB and subsequent downstream proinflammatory processes in a stimulus-specific manner. While AKBI mice demonstrated identical levels of hepatic NF-κB activation in response to endotoxin, a significantly reduced level of hepatic NF-κB activation was observed in AKBI mice after liver ischemia/reperfusion (I/R) injury. This reduced level of NF-κB activation in AKBI mice after liver I/R also correlated with decreased induction of serum TNF-α, reduced hepatic inflammation, and increased survival. In contrast, no differences in any of these indicators were observed between AKBI mice and WT littermates after a lethal injection of LPS. Molecular studies suggest that the specificity of IκBα, but not IκBβ, to properly regulate NF-κB induction during the acute phase of I/R injury is due to injury context–specific activation of c-Src and subsequent tyrosine phosphorylation of IκBα on Tyr42. These results demonstrate that IκBα and IκBβ play unique injury context–specific […]

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IκB proteins play an important role in regulating NF-κB induction following a diverse range of environmental injuries. Studies evaluating IκBβ knock-in mice (AKBI), in which the *IκBα* gene is replaced by the *IκBβ* cDNA, have uncovered divergent properties of IκBα and IκBβ that influence their ability to activate hepatic NF-κB and subsequent downstream proinflammatory processes in a stimulus-specific manner. While AKBI mice demonstrated identical levels of hepatic NF-κB activation in response to endotoxin, a significantly reduced level of hepatic NF-κB activation was observed in AKBI mice after liver ischemia/reperfusion (I/R) injury. This reduced level of NF-κB activation in AKBI mice after liver I/R also correlated with decreased induction of serum TNF-α, reduced hepatic inflammation, and increased survival. In contrast, no differences in any of these indicators were observed between AKBI mice and WT littermates after a lethal injection of LPS. Molecular studies suggest that the specificity of IκBα, but not IκBβ, to properly regulate NF-κB induction during the acute phase of I/R injury is due to injury context–specific activation of c-Src and subsequent tyrosine phosphorylation of IκBα on Tyr42. These results demonstrate that IκBα and IκBβ play unique injury context–specific roles in activating NF-κB–mediated proinflammatory responses and suggest that strategies aimed at inhibiting *IκBα* gene expression may be of potential therapeutic benefit in hepatic I/R injury.

Introduction

The NF-κB/Rel family of transcription factors orchestrates a myriad of cellular responses to environmental injuries by controlling a complex network of NF-κB–regulated gene products involved in regulating immune function, inflammation, programmed cell death, and cellular proliferation (1, 2). The activation of NF-κB is tightly controlled by a series of inhibitory proteins (IκBα, IκBβ, and IκBε) that sequester the NF-κB complex in the cytoplasm and prevent it from binding to DNA in the nucleus. The pathway of NF-κB induction in response to proinflammatory stimuli involves activation of the IκB kinase (IKK) complex that phosphorylates both IκBα and IκBβ on serine residues, leading to their ubiquitination and degradation by the 26S proteasome. This process releases NF-κB from the IκB proteins so that it can translocate to the nucleus. Less well-studied alternative pathways of NF-κB by redox stimuli, such as pervanadate, hydrogen peroxide, and hypoxia/reoxygenation (H/R) injury, have been demonstrated to occur through IκBα Tyr42 phosphorylation (3). This phosphorylation event on IκBα displaces NF-κB from the complex in the absence of IκBα ubiquitin-dependent degradation. The mechanism and physiologic relevance of IκBα tyrosine phosphorylation remains somewhat controversial, but it does appear to require Src family kinases, such as p56Lck, ZAP-70, and c-Src (4–7). Additionally, Tyr42 phosphorylation of IκBα occurs within a consensus binding site for the p85 subunit of PI3K and appears to stabilize its affinity for p85 (8). Although it appears clear that IκBα Tyr42 phosphorylation plays a role in NF-κB activation in response to certain redox stimuli in vitro, the physiologic relevance of this pathway remains obscure. In contrast, IκBβ does not have a conserved Tyr42 phosphorylation site, suggesting the potential for divergent functions between IκBα and IκBβ in response to redox-mediated injury.

Although regulation of NF-κB has been extensively studied in the context of IκBα, IκBβ also plays a key role in regulating the temporal expression of NF-κB. Unlike IκBα, which has both nuclear import and export sequences and hence has the ability to remove activated NF-κB from the nucleus, IκBβ does not enter the nucleus and can only bind to NF-κB in the cytoplasm. The time course of activation of IκBα by the IKK complex is also more acute and short-lived in comparison with IκBβ. This unique aspect of IκBα and IκBβ regulation is proposed to be important in the regulation of the onset and longevity of NF-κB activation (9). IκBα, which demonstrates a rapid degradation and resynthesis in response to stimuli such as LPS and TNF-α, is responsible for the acute-phase activation of NF-κB. In contrast, IκBβ, which demonstrates a delayed degradation and resynthesis, is responsible for the persistence of NF-κB activation (10). Beg and colleagues have argued that IκBα is required for the postinduction repression of NF-κB, while IκBβ facilitates the onset of NF-κB activation in IκBα KO fibroblasts (11). Other major differences between IκBα and IκBβ function have been attributed to the altered regulation of their promoters after injury. NF-κB activation can upregulate *IκBα* gene expression in a feedback regulatory loop (9). In contrast, the *IκBβ* gene is not regulated by NF-κB (9). Despite these differences, it has been suggested that IκBα and IκBβ have redundant functions in the context of proinflammatory injuries (12). This concept stems from work with IκBβ knock-in mice (AKBI),

Nonstandard abbreviations used: alanine aminotransferase (ALT); electromobility shift assays (EMSA); glutathione *S*-transferase (GST); hypoxia/reoxygenation (H/R); IκB kinase (IKK); IκBβ knock-in mice (AKBI mice); ischemia/reperfusion (I/R); myeloperoxidase (MPO); phorbol 12-myristate 13-acetate (PMA); phytohemagglutinin (PHA).

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in which the *IκBα* gene was replaced with the *IκBβ* cDNA. In this model, the regulation of the transgenic *IκBβ* gene was under the direct control of the IκBα promoter. Studies evaluating AKBI mouse fibroblasts and thymocytes have demonstrated that NF-κB induction to proinflammatory stimuli, such as phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA), and TNF-α, is identical to induction to WT mice. In these studies, the major difference between their functions is attributed to altered transcriptional regulation. However, no work evaluating NF-κB–mediated injury responses in intact animals has been reported using this model.

In contrast to proinflammatory stimuli, the influence of NF-κB activation after ischemia/reperfusion (I/R) injury is less understood. Because it is a proinflammatory transcription factor, activation of NF-κB triggers upregulation of cytokines (e.g., TNF-α, IL-1) and adhesion molecules (e.g., ICAM-1) after I/R injury. These cytokines and adhesion molecules act to recruit neutrophils to the damaged organ (13, 14). As an antiapoptotic factor, NF-κB activation also has been suggested to reduce organ damage following H/R (15), partial hepatectomy (16), and I/R (17). Therefore, NF-κB may have both proinflammatory and antiapoptotic effects that influence organ damage after I/R injury. For example, in a xenotransplantation model, inhibition of NF-κB activation by overexpression of IκBα or truncated p65 (p65RHD) reduced inflammatory response and protected the organ from injury (18). In contrast, severe liver degeneration and enhanced apoptotic response to TNF-α have been observed in both knockout mice and fibroblasts lacking IKKβ (19, 20). Furthermore, NEMO/IKKγ-deficient mice also demonstrate severe liver degeneration (21), a result that supports the importance of NF-κB in hepatocellular survival. In adult animals, inhibition of NF-κB by overexpression of a dominant negative IκBα before partial hepatectomy led to massive apoptosis and liver dysfunction (22). Reconciling the proinflammatory and antiapoptotic functions of NF-κB has led to the more recent hypothesis that the temporal pattern of NF-κB activation is an important variable in determining its overall effect (23). To this end, a recent study suggests that although early activation of NF-κB after injury is responsible for the onset of inflammatory response, late-phase activation of NF-κB is required for the resolution of inflammation by expression of anti-inflammatory genes (24). Hence, it has been suggested that the repertoire and temporal activation patterns of numerous transcriptional factors work in concert to determine net effects of NF-κB activation (1).

In the present study, we have explored the functional differences between IκBα and IκBβ using a transgenic AKBI mouse model of liver I/R injury. Our focus on the liver arises from the depth of knowledge on NF-κB function in this organ during development, inflammation, regeneration, and injury. In AKBI mice, the *IκBα* gene is replaced by the *IκBβ* cDNA, so no endogenous IκBα is expressed. Expression of the transgenic *IκBβ* loci is under the direct control of the *IκBα* endogenous promoter. In contrast to *IκBα* KO mice, which have a lethal phenotype, AKBI mice provide an ideal model for assessing the role of IκBα in adult animals, since they develop normally and have regular life spans. Our results demonstrate that AKBI mice and heterozygous littermates respond identically to a lethal intravenous dose of LPS in terms of survival, hepatic NF-κB activation, TNF-α secretion, and liver injury. Both AKBI and WT mouse models of LPS injury also similarly activated the IKK pathway in the liver. In contrast, AKBI mice demonstrate reduced levels of hepatic NF-κB activation after liver I/R that correlate with decreased induction of serum TNF-α, reduced hepatic inflammation, and increased survival. After I/R injury to the liver, c-Src activation predominated with minimal changes in IKK activity. The activation of c-Src after I/R injury led to substrate-specific tyrosine phosphorylation of IκBα but not IκBβ. Furthermore, expression of WT IκBα, but not the IκBα (Y42F) mutant, was capable of rescuing the AKBI phenotype in mouse primary hepatocytes and embryonic fibroblasts after H/R. These results suggest that IκBα has unique injury context–specific biologic properties that cannot be substituted for by IκBβ.

Methods

Mouse model of lobar I/R injury. AKBI transgenic mouse embryos were a gift of R. Bravo (Department of Oncology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey, USA) (12). Partial lobar liver I/R injury was performed, as previously described (25). Briefly, AKBI, WT, and heterozygous littermates were anesthetized with ketamine/xylazine and injected with heparin (100 μg/kg body weight, i.v.) to prevent blood clotting during liver ischemia. The blood supply to the medial, largest lobe of the liver was occluded for 60 minutes by placing a microaneurysm clamp at its base. Following ischemia, the clip was removed and restored blood flow to the liver was observed. The incision was sutured and the animals were returned to their cages for various times of reperfusion. Sham-operated animals were anesthetized and heparinized, and a laparotomy was performed without clamping of the liver. Animal surgeries and handling were performed in accordance with the NIH Animal Guidelines. For electromobility shift assays (EMSA), the medial lobe of the liver was harvested 1 hour after reperfusion, and a nuclear extract was prepared immediately. For ELISA assays, serum samples were collected from blood by cardiac puncture at 3 hours after reperfusion, at which time animals were sacrificed. For survival analysis, animals were monitored for 14 days, after which time the experiment was terminated and the animals sacrificed.

LPS treatment. LPS (prepared from *Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, Missouri, USA) was diluted in PBS to a final concentration of 125 μg/ml. Mice were then injected with LPS (1 or 4 μg/g body weight i.v. for EMSA or survival analysis, respectively). Liver samples were collected 1 hour after treatment, and nuclear extracts were prepared in an identical fashion to that for I/R samples. Serum samples were collected 3 hours after LPS injection, as described above. For survival analysis, animals were monitored for 14 days, after which time the experiment was terminated and the animals sacrificed.

EMSA and Western blot analysis. Whole-cell lysate and nuclear extracts were generated from the liver, as previously described (25). Liver samples from animals that had undergone I/R were harvested from the ischemic lobe. For LPS studies, the entire liver was harvested. For Western blotting, 10 μg of whole-cell lysate was separated on a denaturing 12% SDS-PAGE gel and then transferred to HybondC nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, New Jersey, USA) following standard protocol (25). Ab's against IκBα, IκBβ, and T7 tag were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). EMSA samples were prepared by sucrose centrifugation, and NF-κB DNA binding was examined using an NF-κB–specific oligonucleotide (Promega Corp., Madison, Wisconsin, USA). The oligo sequence was as follows: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. A 5-μg aliquot of nuclear extract was used in each EMSA assay following standard protocols (25).

TNF-α ELISA measurements. To quantify TNF-α protein levels in serum, we harvested serum samples 3 hours after reperfusion by cardiac puncture at the time mice were sacrificed. Control samples were collected at the same time without I/R or LPS treatment. Samples

were standardized to 1 mg/ml protein concentration, and TNF-α levels were measured using a mouse ELISA kit (Pharmingen, Palo Alto, California, USA) following the manufacturer's protocols. To quantify TNF-α mRNA by real-time PCR, we harvested liver samples from sham-operated mice or mice treated with I/R. Total RNA was then prepared using an RNeasy RNA purification kit (QIAGEN Inc., Valencia, California, USA). Using reverse transcriptase (Invitrogen Corp., Carlsbad, California, USA), 5 μg of total RNA were reverse transcribed into cDNA. Real-time PCR was performed using TaqMan Pre-Developed Assay Reagents for mouse *TNF-α* gene expression (Perkin-Elmer Applied Biosystems, Foster City, California, USA).

Histopathology and assessment of liver injury and neutrophil infiltration. Organ histopathology was assessed in AKBI and WT mice after I/R at 18 hours after reperfusion. Organs evaluated for histopathology included the lung, liver, kidney, spleen, and heart. Liver samples were also harvested 18 hours after I/R injury, and myeloperoxidase (MPO) activity was analyzed as an index of neutrophil recruitment using a method described by Schierwagen et al. (26). Samples were homogenized in PBS and centrifuged for 20 minutes at 14,000 *g* at 4°C. The pellet was resuspended with 1% hexadecyltrimethyl-ammonium bromide (Sigma-Aldrich) in 80 mM sodium phosphate, 5 mM EDTA, pH 5.4. The samples were further disrupted by sonication for 10 seconds and then freeze-thawed three times. After heating for 2 hours at 60°C, samples were centrifuged at 15,000 *g* for 15 minutes at 4°C, and the supernatant was used for MPO assays. Reactions were performed by mixing samples with a solution of 1.6 mM 3,3′,3,5′-tetramethylbenzidine (Sigma-Aldrich) and adding hydrogen peroxide to a final concentration of 1 mM. The absorbance at 650 nm was measured immediately for 5 minutes, and the rate of change in absorbance was used to calculate units of MPO activity per gram of tissue using a standard curve. Serum alanine aminotransferase (ALT) was assessed as an index of liver injury after 60 minutes of ischemia and 0, 3, 6, and 18 hours of reperfusion. Blood was collected by retro-orbital bleeding, and ALT levels were assessed using a microkinetic assay as previously described (25). Briefly, the ALT diagnostic kit was used according to the manufacturer's instructions (Sigma-Aldrich), and the change in OD_{340nm} was assessed in a 96-well microtiter plate reader.

Production of primary mouse hepatocytes and H/R experiments. Hepatocytes were isolated from 25- to 30-g AKBI or heterozygote mice as previously described with minor modifications (27, 28). Mice were anesthetized with 0.2 ml nembutanol. The liver was perfused through the portal vein first with Perfusion Buffer Solution and then with Liver Digest Medium (both from Life Technologies Inc., Gaithersburg, Maryland, USA) at a flow rate of 4–6 ml/min for 5 minutes. The liver was then quickly dispersed and filtered through a sterile 100-μm mesh. Hepatocyte suspensions were then centrifuged at 50 *g* for 3 minutes and then resuspended to a density of 5×10^6 cells/ml in DMEM. After Percoll centrifugation at 50 *g* for 10 minutes, viable hepatocytes in the pellet were washed three times and then plated on collagen-coated tissue culture plates in DMEM with 10% calf serum and 100 μg/ml penicillin and streptomycin. After overnight culture, the medium was replaced with F-12 medium containing insulin (10 μg/ml), dexamethasone (67 ng/ml), EGF (50 ng/ml), luteotropin (20 units/liter), linoleic acid (500 μg/ml), transferrin (10 μg/ml), triiodothyronine (67.3 ng/ml), penicillin (100 units/ml), and streptomycin (0.1 mg/ml).

AKBI or heterozygote hepatocytes (1×10^6) were transfected with 4 μg of WT IκBα or 4 μg of the IκBα(Y42F) expression plasmid, together with 1 μg NF-κBLuc reporter plasmid (29). Transfection was performed using Lipofectamine Plus (Life Technologies Inc.) according to the manufacturer's protocol 24 hours prior to treatment with LPS or H/R. H/R experiments were performed as previously described (30). Briefly, a hypoxia medium or reoxygenation medium was produced by equilibrating DMEM (without glucose or FBS) in 95% N_2/5% CO_2 and 95% O_2/5% CO_2, respectively. Hypoxia was induced by incubating cells with hypoxia medium at 37°C for 18 hours in an airtight chamber equilibrated with 5% CO_2 and 95% N_2. The hypoxia medium was then replaced with a reoxygenation medium, and cells were further incubated for 5 hours at 37°C in a chamber flushed with 5% CO_2 and 95% O_2. Samples were harvested 5 hours after reoxygenation for NF-κB luciferase assays using manufacturer's protocols (Promega). Briefly, 5 μg of protein from each sample were assayed for luciferase activity in a luminometer, and luciferase activity was assessed as relative light units.

c-Src and IKK in vitro kinase assays and assessment of IκBα and IκBβ phosphorylation. To evaluate tyrosine phosphorylation of IκBα or IκBβ after liver I/R, 200 μg of whole-liver lysate were immunoprecipitated using 2 μg of an IκBα Ab (Santa Cruz Biotechnology Inc.). Tyrosine phosphorylation was then determined by Western blot analysis by using an anti-phosphotyrosine Ab (Santa Cruz Biotechnology Inc.). The ability of c-Src or the IKKα/β complex to phosphorylate IκBα and/or IκBβ after I/R injury or LPS treatment was evaluated using radioactive in vitro kinase assays, as previously described (30). Briefly, 500 μg of protein were immunoprecipitated from liver lysates with Ab's to c-Src or to IKKα/β (Santa Cruz Biotechnology Inc.). Then, 1 μg of glutathione *S*-transferase-IκBα (GST-IκBα) or GST-IκBβ fusion protein was then added as a substrate in the presence of 10 μl kinase buffer (40 mM HEPES, 1 mM β-glycerophosphate, 1 mM nitrophenolphosphate, 1mM Na_3VO_4, 10 mM $MgCl_2$, 2 mM DTT, 0.3 mM cold ATP, and 10 μCi [γ-^{32}P]ATP) and was incubated at 30°C for 30 minutes. Samples were then loaded onto a 10% SDS-PAGE gel, and proteins were transferred to nitrocellulose membrane for autoradiography and Western blotting against anti-GST Ab's. Direct evaluation of tyrosine phosphorylation of GST-IκBα by c-Src in WT and ABKI mouse liver samples after I/R injury was evaluated using nonradioactive in vitro c-Src kinase assays. These assays were performed in an identical manner to the radioactive kinase assay described above, except for the replacement of [γ-^{32}P]ATP with nonradioactive ATP. Tyrosine phosphorylation on GST-IκBα was then evaluated by Western blotting using an antiphosphotyrosine Ab (Santa Cruz Biotechnology Inc.).

Statistical analyses. Statistical analyses were performed using the software GraphPad Prism Version 3.0a (GraphPad Software, San Diego, California, USA). TNF-α measurements were analyzed using the Student's *t* test, and the survival rate was analyzed using the log-rank test. Statistical significance was set at $P < 0.05$. For survival studies following LPS challenge, 20 sets of age-matched littermate male mice were evaluated with AKBI or WT genotypes. For survival studies following liver I/R injury, 3 female and 15 male age-matched mice were evaluated in each AKBI and WT genotype group. Four mice from the AKBI and four mice from the WT groups died during the surgery procedure and were excluded from the survival data.

Results

NF-κB activation in the liver after I/R is significantly reduced in AKBI mice while remaining unaffected after LPS treatment. Using two distinct types of injury (I/R and LPS) that are both dependent on *NF-κB* activation and subsequent TNF-α–mediated proinflammatory responses, we have investigated the functional redundancy of IκBα and IκBβ using a transgenic AKBI mouse model in

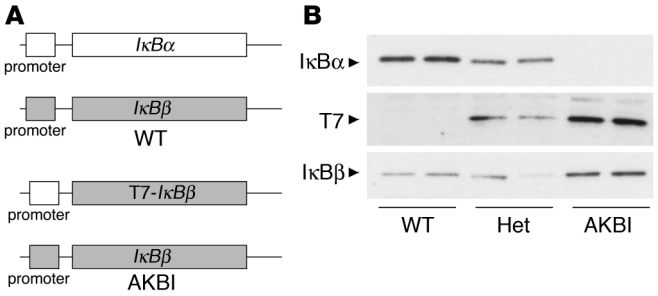

Figure 1
IκB expression patterns in AKBI mice. (**A**) The *IκBα* gene loci in AKBI mice are replaced by the T7-tagged IκBβ cDNA. The *IκBα* endogenous promoter controls the expression of the T7-*IκBβ* transgenic loci in AKBI mice, while the endogenous *IκBβ* remains under the control of its own endogenous promoter. (**B**) A 10-μg aliquot of liver cell lysate, separated by 12% SDS-PAGE, was evaluated by Western blotting using Ab's to IκBα, IκBβ, and T7. Two littermates were evaluated for each group of WT, heterozygous (Het), or AKBI genotypes.

which the *IκBα* gene is replaced by the T7-tagged IκBβ cDNA (Figure 1). To confirm the expression patterns of IκBα and IκBβ in this transgenic model, we evaluated the protein levels of IκBα and IκBβ. Western blot analysis of liver lysates from AKBI, heterozygous, and WT littermates demonstrated the correct expression patterns: IκBα, IκBβ, or the T7-tagged IκBβ in the various genotypes (Figure 1B). Previous reports using isolated fibroblasts from this AKBI model have demonstrated that TNF-α or PMA treatment similarly induce NF-κB activation in AKBI and WT littermates. To establish that in vivo results correlate with this previous in vitro study, we sought to confirm that IκBα and IκBβ both function similarly to induced NF-κB after an intravenous LPS challenge. To this end, AKBI or WT littermates were challenged with LPS 1 hour prior to assessing NF-κB activation by EMSA. Consistent with the previous reports evaluating proinflammatory stimuli in this model, we observed no significant difference in induction of hepatic NF-κB DNA binding after LPS treatment between the AKBI and WT groups of treated animals (Figure 2A). These data support the notion that IκBα and IκBβ have redundant functions in terms of mediating NF-κB activation after proinflammatory stimuli, such as TNF-α or LPS treatment.

The notion that IκBα and IκBβ have totally redundant functions is challenged by the observations that proinflammatory stimuli or redox-mediated I/R (or H/R) injury appears to activate NF-κB through independent mechanisms that control either IKK-mediated serine phosphorylation of IκBα or Src family kinase–mediated tyrosine phosphorylation of IκBα, respectively. IκBβ retains consensus serine phosphorylation sites, which are similar to the IKK targets for serine phosphorylation in IκBα. However, no consensus tyrosine phosphorylation sites exist in IκBβ, nor has this molecule been demonstrated to be a substrate for tyrosine phosphorylation. Thus, we hypothesized that IκBα and IκBβ may play unique roles in regulating NF-κB following I/R injury and that tyrosine phosphorylation of IκBα is compulsory for NF-κB activation in this setting. In support of this hypothesis, our results demonstrated significantly attenuated levels of NF-κB activation in AKBI mice as compared with WT mice following partial lobar I/R injury to the liver (Figure 2B). These results suggested that in the context of liver I/R, IκBβ cannot functionally replace IκBα to induce NF-κB. Taken together, the results from both LPS and I/R treatment studies in this AKBI model suggest that IκBα and IκBβ have injury context–dependent functional differences in their ability to activate NF-κB.

TNF-α production in AKBI mice is significantly reduced following I/R, but not LPS-mediated injury. TNF-α release by Kupffer cells is an important hepatic signal involving inflammatory responses to both I/R (31, 32) and LPS injury (33, 34). TNF-α released by the liver after I/R or LPS exposure acts to signal an NF-κB–dependent proinflammatory cascade that results in massive neutrophil recruitment. Having demonstrated that IκBα and IκBβ differentially regulate NF-κB activation after LPS or I/R injury to the liver, we next sought to test how TNF-α secretion was affected by the altered NF-κB responses seen in AKBI mice following each of these stimuli. Consistent with our data on NF-κB activation, no significant changes in serum TNF-α levels were seen in AKBI and WT mice following LPS exposure (Figure 3A). In contrast, after I/R injury, AKBI mice demonstrated a reduction in serum TNF-α levels that was more than twofold lower than in WT littermates (Figure 3B). Real-time PCR analysis of liver TNF-α mRNA substantiated findings of lower TNF-α levels in AKBI livers (Figure 3C). Hence, following I/R injury, the decreased ability of the AKBI mice to activate hepatic NF-κB correlated with a concomitant reduction in TNF-α transcription and secretion. However, following LPS exposure, sustained activation of NF-κB in AKBI mice correlated with a sustained ability to produce TNF-α.

Survival and hepatic inflammation are significantly altered in AKBI mice following I/R, but not LPS-mediated injury. Having demonstrated that both NF-κB activation and TNF-α production were significantly reduced at acute time points after post-I/R injury in our AKBI mouse model, we reasoned that during later phases of injury, changes in TNF-α might be reflected in a reduced capacity of the liver to recruit neutrophils. To this end, we followed a cohort of AKBI and WT lit-

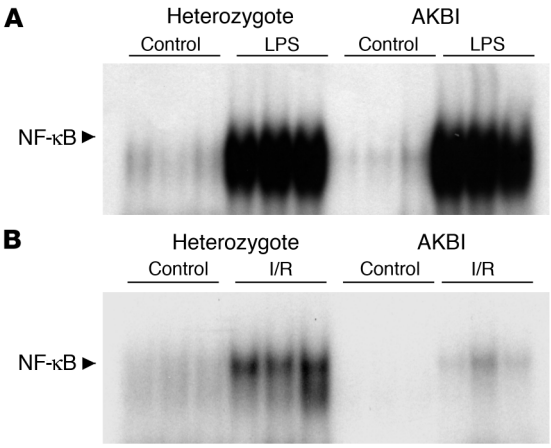

Figure 2
NF-κB activation in the liver following I/R is significantly reduced in AKBI mice but remains unaltered following an LPS challenge. (**A**) AKBI mice or heterozygous littermates were challenged with LPS (1 μg/g body weight, i.v.), and nuclear extracts from the liver were prepared at 1 hour after LPS injection. PBS-injected mice were also harvested as controls. (**B**) AKBI mice or heterozygous littermates were challenged with I/R, and nuclear extracts were prepared at 1 hour after reperfusion. Sham-operated mice were used as controls. For each sample, 5 μg of nuclear protein were evaluated by EMSA ($n = 3$ animals in each treatment group) to determine NF-κB DNA-binding activity. An arrow to the left of the gel indicates the presence of the induced p65/p50 heterodimer of NF-κB.

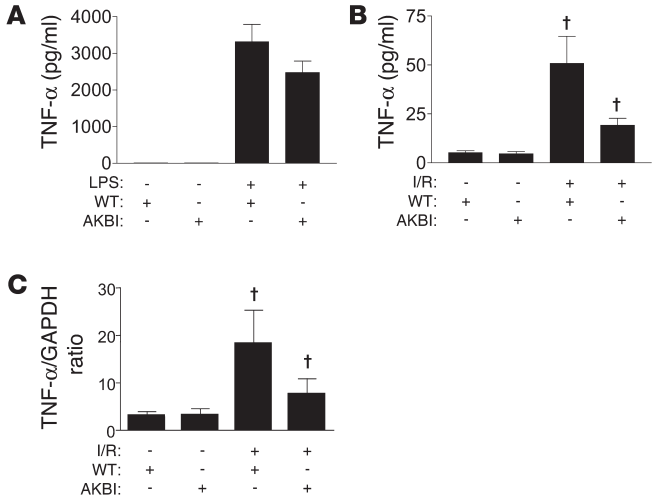

Figure 3
TNF-α production following liver I/R, but not an LPS challenge, is significantly reduced in AKBI mice. (**A**) AKBI mice and WT littermates were challenged with LPS (1 μg/g body weight, i.v.), and serum samples were harvested by cardiac puncture at 3 hours after LPS injection. Serum samples from PBS-injected mice were collected at the same time as controls. (**B**) AKBI mice and WT littermates underwent liver I/R, and serum samples were harvested by cardiac puncture at the end of 3 hours of reperfusion. Sham-operated mice were used as controls. Serum samples were further standardized to 1 mg/ml protein concentration, and an ELISA was performed to quantify TNF-α levels. (**C**) Liver TNF-α mRNA expression was quantified at 3 hours after reperfusion injury by real-time PCR in AKBI and WT mice littermates. Sham-operated mice were used as controls. TNF-α mRNA expression was normalized to GAPDH mRNA levels. Values in **A**–**C** represent the mean (± SEM) for $n = 4$ independent animals in each group. Differences marked by a dagger in **B** and **C** are statistically significant by the Student's t test ($P < 0.05$).

termates to later time points that encompassed the neutrophil-predominant subacute inflammatory responses normally seen at 16–20 hours after liver I/R or LPS challenge. Indeed, histopathology of the liver at 18 hours after I/R demonstrated a significant reduction in recruited neutrophils to the liver in AKBI compared with WT littermates (Figure 4A). As an index for neutrophil recruitment in the liver, we also assessed MPO levels in liver lysates after I/R injury. Results from this analysis substantiated histopathologic observations and demonstrated significantly lower levels of MPO activity in AKBI livers than in WT littermates after I/R (Figure 4B). The reduced level of neutrophil recruitment in AKBI mice also correlated with reduced serum ALT levels as an index of liver I/R injury (Figure 4C).

These analyses demonstrating reduced liver injury and inflammation in AKBI mice following liver I/R correlated with a reduction in NF-κB activation. Notably, this decreased inflammation in the liver of AKBI mice following I/R injury led to a significantly increased sur-

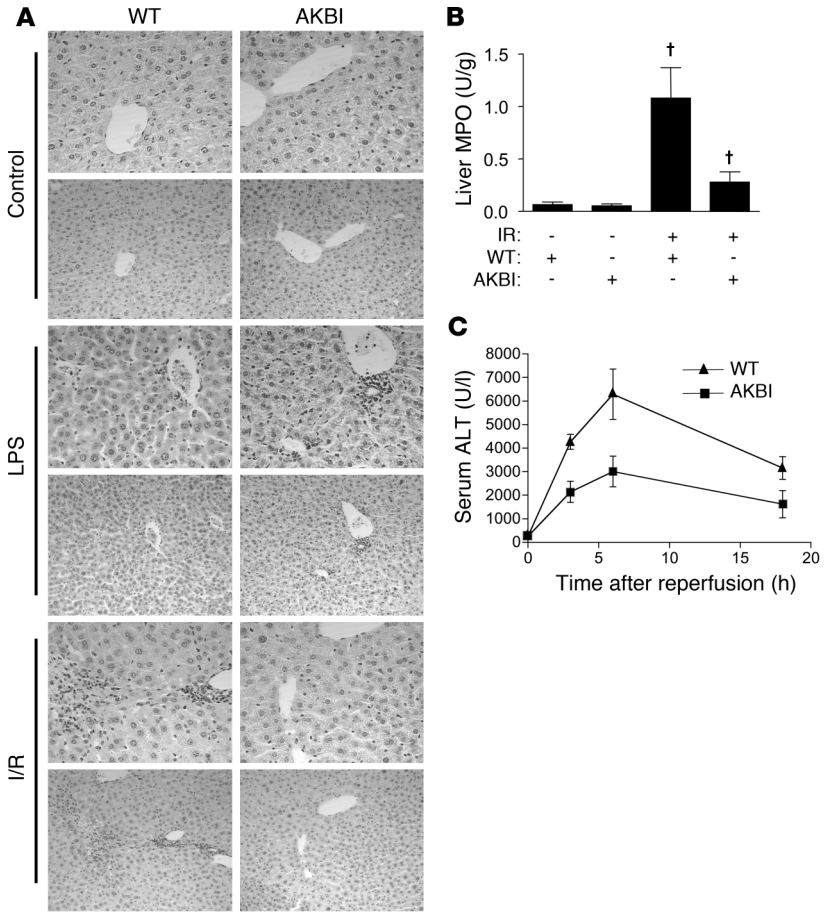

Figure 4
Neutrophil recruitment and liver dysfunction following liver I/R, but not LPS challenge, is significantly attenuated in AKBI mice. (**A**) Liver tissues from ABKI and WT littermates were evaluated for histopathology in paraffin sections following LPS or I/R treatment. Livers from LPS-injected (4 μg/g body weight, i.v.) or I/R-treated (1 hour ischemia) mice were harvested at 18 hours after LPS treatment or reperfusion, respectively. Tissue samples were then fixed with 10% neutral formalin and embedded in paraffin for sectioning. Photomicrographs depict high-power (×400) and lower (×100) power representative photomicrographs for both AKBI and WT littermates for the indicated conditions. The high-power photomicrograph is an enlargement of the lower power field given for each example. (**B**) Hepatic neutrophil accumulation in WT and AKBI mice at 18 hours after reperfusion was quantified by measuring liver MPO activity. Values represent the mean (± SEM) for $n = 4$ independent animals in each group. (**C**) AKBI mice were compared to WT mice for liver function following I/R at the indicated time points. Values depict ALT levels (mean ± SEM) as an index for liver injury from $n = 4$ animals for each experimental point. Differences marked by daggers in **B** are statistically significant by the Student's t test ($P < 0.05$). ALT profiles were also significantly different between AKBI and WT mice ($P < 0.05$) as assessed by ANOVA.

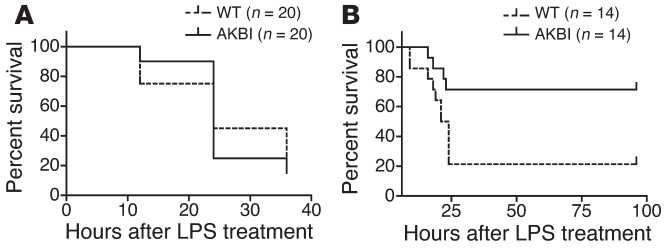

Figure 5
AKBI mice have improved survival following I/R injury. AKBI or WT littermates either (**A**) received a lethal dose of LPS (4 μg/g body weight, i.v.) or (**B**) underwent partial lobar liver I/R. Survival was assessed for 14 days. Only times of survival are given for which deaths occurred. No deaths occurred past the plotted times. Survival curves were significantly different between AKBI and WT mice ($P < 0.05$) as assessed using the log-rank test.

vival rate in mice 14 days after I/R ($n = 14$, $P < 0.05$) (Figure 5B). To confirm that WT mice were dying predominantly from liver-associated inflammation, we also evaluated the histopathology of the lung, spleen, kidney, and heart. No significant abnormalities or differences were observed between AKBI and WT mice in the histology of these organs after I/R injury (data not shown). In contrast to our observations of decreased inflammation and increased survival of AKBI mice after liver I/R, LPS-induced hepatic inflammation and death occurred at indistinguishable frequencies in AKBI and WT littermates (Figures 4A and 5A). In summary, our comparative data on LPS- and I/R-associated liver injuries strongly suggests that IκBα and IκBβ function similarly to regulate NF-κB–mediated proinflammatory responses after LPS challenge but have divergent abilities to carry out similar inductive cascades after I/R injury.

Hepatic I/R induces c-Src–mediated tyrosine phosphorylation of IκBα, but not IκBβ. Previous studies have demonstrated that partial lobar liver I/R injury induces NF-κB activation in association with IκBα tyrosine phosphorylation and an absence of IκBα degradation (25). The absence of IκBα degradation typically associated with the canonical proinflammatory IKK-mediated pathway of NF-κB activation was used to infer that these two types of injury may activate NF-κB through different sets of signal intermediates. From the standpoint of the present study, the conserved tyrosine phosphorylation site (Tyr42) exists only in IκBα. Thus, AKBI mice that lack IκBα and this conserved site of tyrosine phosphorylation provide a unique model for assessing the selective functions of IκBα and IκBβ in the context of I/R injury. Hence, we hypothesized that the difference in NF-κB activation after liver I/R between AKBI and WT mice might be due to the need for tyrosine phosphorylation of IκBα to activate NF-κB after this type of injury. To establish biochemically that IκBα and IκBβ are differential substrates for tyrosine kinases after I/R injury to the liver, we directly evaluated the extent of IκBα and IκBβ tyrosine phosphorylation after I/R injury. Consistent with our previous findings, IκBα tyrosine phosphorylation significantly increased at 1 hour following I/R injury in WT mice (Figure 6A). In contrast, no tyrosine phosphorylation of IκBβ was detected following I/R (Figure 6B). These findings support the notion that IκBα, but not IκBβ, is a direct substrate for tyrosine kinases following I/R injury.

Given the previous reports associating redox activation of c-Src family kinases following H/R in cell lines with tyrosine phosphorylation of IκBα (4, 30, 35), we further extended our hypothesis to test whether c-Src activation following liver I/R acts in a substrate-specific manner to tyrosine-phosphorylate GST-IκBα, but not GST-IκBβ. Results from these in vitro kinase assays demonstrated that immunoprecipitated c-Src was activated to phosphorylate GST-IκBα by 30–60 minutes after hepatic reperfusion of WT animals (Figure 7A). In contrast, immunoprecipitated c-Src from these same samples was not able to phosphorylate GST-IκBβ (Figure 7B). These findings, which for the first time demonstrate substrate selectivity in the ability of c-Src to phosphorylate IκBα, but not IκBβ, support the hypothesis that the AKBI phenotype (i.e., reduced NF-κB induction following I/R) is due to the inability of IκBβ to respond to I/R-induced c-Src signals. We also recognized that differences seen in the ability of this c-Src pathway to activate NF-κB in AKBI and WT mice could also be due to impaired activation of the c-Src pathway in AKBI mice following I/R. To rule out this possibility, we directly compared the extent of c-Src activation following I/R in both genotypes of mice. Results from this study clearly demonstrated that the abilities of immunoprecipitated c-Src from I/R liver lysates of AKBI and WT mice to phosphorylate GST-IκBα in vitro were similar (Figure 7C). Hence, c-Src activation was not impaired in AKBI mice. In summary, these experiments clearly demonstrate the activation of c-Src following I/R injury to the liver and the preferential targeting of IκBα tyrosine phosphorylation by c-Src.

LPS and I/R liver injury induce IKK activation with differing kinetics. NF-κB activation can occur through at least two mechanisms. Following H/R or I/R injury, activation of protein tyrosine kinases, such as c-Src, can mediate the tyrosine phosphorylation of IκBα on Tyr42, leading to the mobilization of NF-κB to the nucleus in the absence of IκBα degradation. In contrast, proinflammatory stimuli, such as LPS and TNF, activate NF-κB through IKK-mediated serine phosphorylation of both IκBα and IκBα, promoting

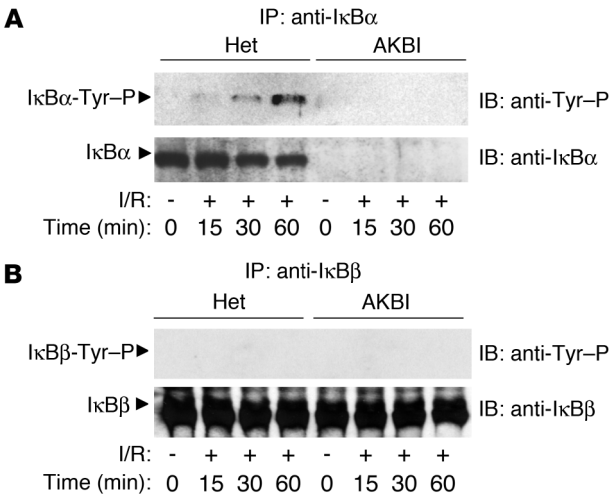

Figure 6
IκBα tyrosine phosphorylation in the liver following I/R is significantly reduced in AKBI mice. AKBI mice or heterozygous (Het) littermates were challenged with I/R, and whole-cell liver extracts were prepared at 15, 30, or 60 minutes after reperfusion. Sham-operated mice were used as controls. Aliquots of 300 μg of whole-cell lysate protein were immunoprecipitated (IP) with (**A**) anti-IκBα Ab or (**B**) anti-IκBβ Ab, followed by Western immunoblotting (IB) with an anti-phosphotyrosine Ab (anti–Tyr-P) to detect tyrosine phosphorylation on IκBα or IκBβ (top blot in each panel). Western blots were stripped and reprobed with anti-IκBα or anti-IκBβ Ab's as loading controls (bottom blot in each panel).

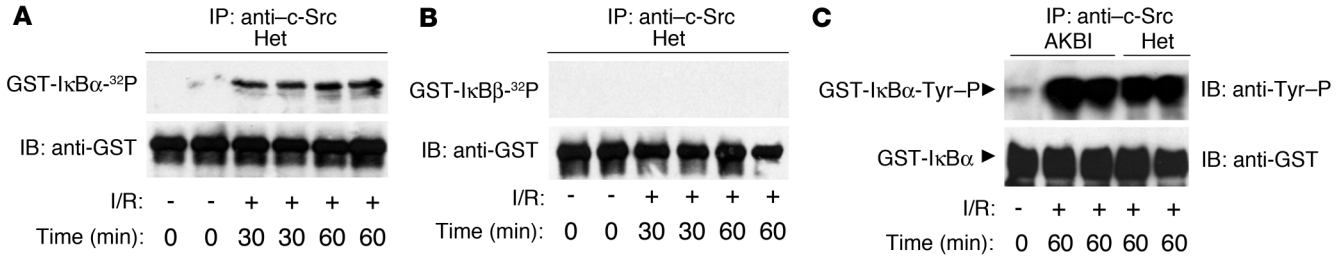

Figure 7
The tyrosine kinase c-Src is able to phosphorylate IκBα, but not IκBβ, in the liver following I/R, and c-Src activation following I/R is unaltered in AKBI mice. Heterozygous (Het) littermates were challenged with liver I/R, and whole-cell liver extracts were prepared at 30 or 60 minutes after reperfusion. Sham-operated mice were used as controls. Aliquots of 300 μg of whole-cell lysate protein were immunoprecipitated (IP) with a c-Src Ab. The ability of immunoprecipitated c-Src to directly phosphorylate (**A**) GST-IκBα or (**B**) GST-IκBβ fusion protein was then evaluated in an in vitro kinase assay with [γ-^{32}P]ATP. Kinase reactions were evaluated by SDS-PAGE, followed by transfer to a nylon membrane. Membranes were first exposed to film (top panels) and then immunoblotted (IB) with anti-GST Ab and ECL detection to confirm equal loading (bottom panel). (**C**) The c-Src activities in AKBI mice and heterozygous littermates were compared using a cold in vitro kinase assay. The ability of immunoprecipitated c-Src to directly tyrosine-phosphorylate GST-IκBα fusion protein was evaluated by immunoblotting with anti-phosphotyrosine Ab (anti–Tyr-P; top panel) using ECL detection. Equal loading of GST-IκBα fusion protein was confirmed by Western blotting of the same sample set in a second gel and immunoblotting with an anti-GST Ab (bottom panel).

ubiquitin-dependent degradation of IκB. We hypothesized that these two fundamental pathways of NF-κB activation account for the observed stimuli-specific differences seen (i.e., NF-κB activation and inflammatory responses) in the livers of ABKI mice in response to LPS and I/R.

To establish more conclusively that LPS or I/R injury to the liver initiates stimuli-specific responses involving IKK or c-Src, respectively, we compared the activated state of the IKK complex in the liver following these two stimuli. Results from these in vitro kinase experiments demonstrated maximal IKK activation by 1 hour after LPS injection in both AKBI mice and heterozygous littermates that declined by 3 hours (Figure 8A). The indistinguishable IKK activation pattern between AKBI and heterozygous littermates was consistent with our findings that NF-κB activation was also similar following LPS treatment in these two genotypes of mice. In contrast, a significantly lower level of IKK activation was seen following I/R injury to the liver in both genotypes of mice at 1 hour after reperfusion (Figure 8B). However, IKK activity following I/R increased by 3 hours after reperfusion. These findings are consistent with IKK playing only a minor role in regulating the phosphorylated state of IκBα during the acute phases of I/R injury while taking on a more predominant role during the subacute stages of NF-κB activation, which involve responses to cytokines produced during the acute phase (Figure 8B). In support of this hypothesis, the onset of IKK activation was slightly slower in AKBI mice that have less severe subacute inflammatory responses. Together with the confirmed specificity of c-Src to use IκBα, but not IκBβ, as a substrate following I/R injury, these findings support the notion that IKK-mediated serine or c-Src–mediated tyrosine phosphorylation of IκBα provides injury context–specific signals necessary for the activation of NF-κB.

In vitro restoration of WT IκBα, but not the IκBα(Y42F) mutant, restores NF-κB activation in AKBI mouse primary hepatocytes following H/R. One hypothesis capable of explaining the observed phenotypic differences between AKBI and WT mice in response to liver I/R, but not LPS, injury suggests that IκBα possesses injury context–specific functions in the setting of I/R injury that cannot be replaced by IκBβ. Such a hypothesis assumes that Tyr42 phosphorylation of IκBα is necessary for NF-κB activation following I/R injury. To prove this hypothesis, we next sought to demonstrate that expression of WT IκBα, but not the IκBα(Y42F) mutant, could functionally restore NF-κB inducibility in a setting similar to H/R. We have previously demonstrated that H/R in cell lines can activate NF-κB in a fashion similar to that of I/R through c-Src–mediated IκBα tyrosine phosphorylation (30). With the goal of further explaining injury context–specific functions of IκBα and IκBβ in our AKBI model, we developed an in vitro model to study NF-κB activation in primary mouse hepatocytes derived from heterozygote or AKBI mice.

Studies using LPS or H/R injury in this model confirmed our in vivo findings by demonstrating that NF-κB responses to LPS were similar in AKBI and heterozygote hepatocytes, but substantially different in response to H/R injury (Figure 9). Transcriptional activa-

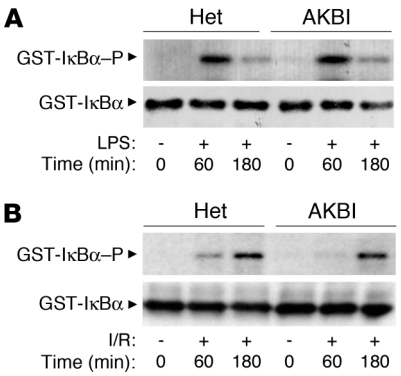

Figure 8
LPS and I/R liver injury induce IKK activation with differing kinetics. AKBI or heterozygous (Het) littermates were challenged with LPS (1 μg/g body weight, i.v.) or I/R, and whole-cell extracts were prepared at 60 and/or 180 minutes after reperfusion or after LPS treatment. The IKK complex was immunoprecipitated with an anti-IKKα/β Ab and used in an in vitro kinase assay. The ability of immunoprecipitated IKK to directly phosphorylate GST-IκBα following (**A**) LPS treatment or (**B**) I/R in AKBI mice or heterozygous mice was then compared in the presence of [γ-^{32}P]ATP. Kinase reactions were evaluated by SDS-PAGE, followed by transfer to a nylon membrane. Top membranes in each panel were exposed to film, and then bottom membranes in each panel were immunoblotted with an anti-GST Ab to confirm equal loading (bottom panel).

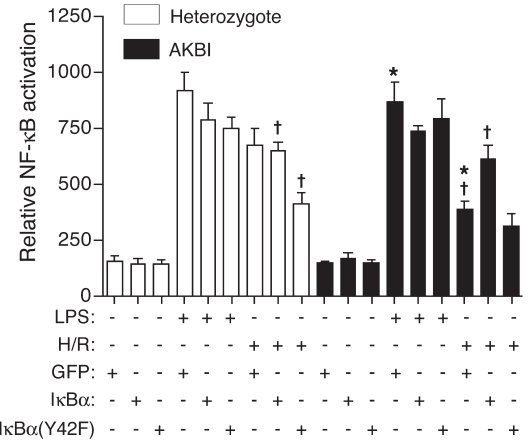

Figure 9

Ectopic expression of IκBα, but not IκBα(Y42F), reconstitutes NF-κB activation in AKBI mouse primary hepatocytes. Heterozygote or AKBI hepatocytes were transfected with IκBα, IκBα(Y42F), or GFP expression plasmids together with a pNF-κBluc reporter plasmid 24 hours prior to LPS or H/R injury. Cells were treated with LPS (1 μg/ml) or H/R (18 hours hypoxia, 5 hours reoxygenation), and whole-cell extracts were harvested 5 hours following reoxygenation or LPS treatment. Luciferase assays were used to evaluate NF-κB transcriptional activation. Values depict relative light units as an index for relative NF-κB activity (mean ± SEM, $n = 6$). Paired comparisons between samples in a given genotype are significant as marked using the Student's t test: †$P < 0.05$; *$P < 0.01$.

tion of NF-κB was significantly ($P < 0.001$) impaired in AKBI as compared with heterozygote hepatocytes following H/R injury. Furthermore, unlike the in vivo models of liver injury, the levels of NF-κB activation in heterozygote hepatocytes were similar following LPS and H/R. This attractive feature of the hepatocyte model permitted a more direct evaluation of injury-specific pathways of NF-κB activation with similar intensities. Most importantly, transfection of a WT IκBα expression plasmid restored NF-κB activation in AKBI hepatocytes following H/R injury to a level seen in heterozygote hepatocytes. In contrast, transfection of a mutant IκBα(Y42F) expression plasmid had no effect on NF-κB activation following H/R in AKBI hepatocytes (Figure 9). Furthermore, transfection of the mutant IκBα(Y42F), but not the WT IκBα, expression plasmid significantly ($P < 0.05$) inhibited NF-κB activation following H/R in heterozygote hepatocytes. In support of the context-specific function of Tyr42 on IκBα, expression of the IκBα(Y42F) mutant had no significant effect on LPS activation of NF-κB in either AKBI or heterozygous mice. Results similar to those seen in primary hepatocytes were also observed with heterozygote and AKBI primary fetal fibroblasts (data not shown). Taken together, these results provide strong evidence that IκBα and IκBβ play injury context–specific roles in activating NF-κB following LPS or I/R injury. The results also show that selective Tyr42 phosphorylation of IκBα imparts specificity to I/R injury responses that induce NF-κB.

Discussion

Transgenic models aimed at dissecting the function of IκB family members have shed considerable light on the NF-κB pathway and its importance in developmental and inflammatory responses (36). IκBα-deficient mice display a lethal neonatal phenotype with extensive dermatitis and granulopoiesis (11, 37). Interestingly, IκBα-deficient mouse fibroblasts retain the ability to induce NF-κB following TNF-α exposure, but also demonstrate a delayed downregulation of NF-κB activation. The altered kinetics in NF-κB activation are now known to be due to the ability of IκBα, but not IκBβ, to remove activated NF-κB from the nucleus (38). IκBε, which is mainly expressed in thymocytes in the spleen, thymus, and lymph nodes, is not required for survival or normal development. However, IκBε-deficient mice experience increased basal and antigen-specific Ab production by B cells (39). Although no IκBβ KO model currently exists, the function of IκBβ has been previously studied in the AKBI mouse model, in which the *IκBβ* cDNA has replaced the *IκBα* gene loci. AKBI mice develop normally, have normal life spans, and show no difference in their abilities to induce NF-κB activation following proinflammatory stimuli (12). Our current study using this AKBI mouse model suggests that the previously reported redundancies in IκBα and IκBβ functions in response to proinflammatory stimuli may not be similar for all types of environmental injuries.

The IκB families of proteins share many similarities in their structures and functions. IκBα and IκBβ are the most similar in structure, with several ankyrin repeats responsible for binding the NF-κB complex and two conserved serine residues that can be phosphorylated by the IKK complex. When the AKBI mouse line was first established, the authors found no difference between AKBI and WT littermates in development and NF-κB responses to PMA/PHA and TNF-α (12). Thus, it was suggested that IκBα and IκBβ functions were redundant. Differences in phenotype between the IκBα-deficient mouse model and this AKBI mouse model were hence thought to be predominantly due to altered regulation of the two IκB promoters (with the *IκBα*, but not *IκBβ*, promoter being autoregulated by NF-κB). Our data evaluating LPS challenge in this AKBI model substantiates these previous findings, suggesting that IκBα and IκBβ have redundant functions in activating NF-κB, TNF-α, and inflammation in the setting of proinflammatory stimuli.

Cumulatively, the data on AKBI mice suggest that for proinflammatory pathways involving NF-κB, IκBα and IκBβ can carry out similar redundant functions. Such findings may not be surprising, since the IKK complex mediates the proinflammatory pathway of NF-κB activation through similar mechanisms of serine phosphorylation on both IκBα and IκBβ. However, this canonical proinflammatory IKK-mediated pathway of NF-κB activation, which acts via the phosphorylation-dependent ubiquitin degradation of both IκBα and IκBβ, is only one of two pathways known to modulate the cytoplasmic IκBα–NF-κB complex. To this end, several stimuli, including H/R, pervanadate, and H_2O_2, have been demonstrated to activate NF-κB through Tyr42 phosphorylation of IκBα in the absence of ubiquitin-dependent degradation (3). This functional regulation is not conserved for IκBβ (3). These findings have suggested that IKK-independent pathways can function under specific redox-mediated stimuli to activate NF-κB. In vivo support of this pathway was first identified following partial lobar I/R in the mouse liver, where NF-κB is activated in the absence of degradation of IκBα and associated with increased IκBα tyrosine phosphorylation (25). Despite these associations of redox injury with increased IκBα tyrosine phosphorylation and its dependence on Src family kinases (4–7, 30), the in vivo functional significance of this pathway of NF-κB activation has remained obscure. However, given that IκBα and IκBβ are not conserved in their ability to be tyrosine-phosphorylated, these findings suggest that AKBI mice might demonstrate altered NF-κB responses to redox-mediated environmental injuries.

Using partial lobar liver I/R as a model for in vivo redox-mediated injury, we sought to test this hypothesis. In contrast with LPS chal-

lenge studies, AKBI mice demonstrated a significantly reduced activation of NF-κB compared with heterozygous littermates following I/R liver injury. This reduced level of NF-κB activation observed in AKBI mice following liver I/R also correlated with decreased induction of serum TNF-α, reduced hepatic inflammation, and increased survival. Given the magnitude of change in survival, and that gross liver necrosis restricted to the ischemic lobe was similar in both AKBI and WT mice, these data indicate that death in the WT animals probably occurred as a result of systemic injury. However, histopathology did not reveal gross abnormalities in the other organs evaluated. These data suggested the hypothesis that IκBα has a unique ability to regulate NF-κB and downstream proinflammatory responses in the context of I/R injury that is not conserved in IκBβ.

Our findings demonstrating that the IKK complex was not substantially activated in either genotype during the acute phase (<1 hour) of I/R injury supported the notion that an alternative independent pathway is likely responsible for NF-κB activation during this phase of injury. Based on previous work evaluating c-Src–mediated activation of NF-κB following H/R injury (30), we confirmed that c-Src was indeed equivalently activated in both AKBI and WT mouse livers during the acute phase (30–60 minutes) of I/R injury. Additional studies demonstrating that c-Src selectively used IκBα, but not IκBβ, as a substrate for tyrosine phosphorylation supported findings of selective tyrosine phosphorylation of endogenous IκBα following I/R in WT but not AKBI mice. Taken together, these findings have for the first time assigned in vivo relevance to c-Src–mediated IκBα tyrosine phosphorylation-dependent pathways of NF-κB activation. Furthermore, studies reconstituting WT and Y42F-mutant IκBα in AKBI and heterozygote hepatocytes conclusively demonstrate the functional requirement for Tyr42 phosphorylation of IκBα in the activation of NF-κB following H/R. Although we cannot presently rule out that the IKK complex also plays a role in transducing c-Src signals to IκBα, these studies do conclusively demonstrate that selective phosphorylation of IκBα on Tyr42 imparts injury context–specific functions in the activation of NF-κB that are not conserved in IκBβ.

The low level of IKK activation in the immediate acute phase of I/R injury does not exclude IKK from playing an important role in liver inflammation and remodeling following I/R. In fact, our studies demonstrating that maximal IKK activation follows c-Src activation implicates IKK in downstream proinflammatory cytokine responses initiated by the redox-dependent activation of c-Src during the initial reperfusion stage. In support of this notion, previous work has also implicated the IKK complex in inflammatory responses following intestinal and cardiac I/R models where IKK activity is elevated (40, 41). Given that the peak of c-Src activation precedes that of IKK activation, the temporal pattern of the two pathways probably plays an important role in regulating inflammation and regeneration to I/R injury.

Differences in the mechanisms of LPS and I/R injury may account for the evolutionarily diverged functions of IκBα and IκBβ in regulating NF-κB activation. For example, the induction of NF-κB following liver I/R injury is regulated by acute redox-activated responses that seem to involve NADPH oxidase (42). Clearance of these superoxides attenuate NF-κB activation and inhibit liver injury following I/R (25). Although TNF-α has been implicated in the hepatic I/R injury process during the subacute phases of injury (43), the levels of TNF-α produced in the setting of I/R injury are much less than those following LPS injury. Proinflammatory stimuli, such as LPS and TNF-α, also activate NF-κB through ligand–receptor interactions in a manner that is different from redox acute activation of NF-κB following I/R injury. Additionally, proinflammatory models of acute inflammation, such as carrageenan-induced pleurisy, have suggested that NF-κB activation plays an important role not only in activating proinflammatory cytokine cascades (such as TNF-α) that lead to inflammation, but also in resolving the resulting inflammation through anti-inflammatory pathways and leukocyte apoptosis (24). When considering injury context–specific mechanisms of NF-κB activation, it is important to appreciate that NF-κB must work in concert with other signaling pathways that ultimately determine the response of a given organ to injury. In the context of I/R injury, c-Src is uniquely positioned to respond to redox-mediated stimuli in a receptor-independent fashion (30, 44). Several groups have identified Src family tyrosine kinases involved in phosphorylating IκBα in addition to c-Src. For instance, spleen tyrosine kinase mediates IκBα tyrosine phosphorylation following hydrogen peroxide treatment in human myeloid KBM-5 cells (45). Thus, c-Src activation following liver I/R may act as a redox sensor for NF-κB activation. Such pathways of NF-κB activation seem to be fundamentally different from those involved in proinflammatory assaults such as LPS, which mediate NF-kB activation through the canonical IKK-mediated pathway.

Evidence in the literature supports both the proinflammatory and antiapoptotic roles of NF-κB in the context of liver injury (22, 46). Since the inductive phase of NF-κB is significantly attenuated in AKBI mice following liver I/R, this model provides a unique venue to evaluate whether NF-κB induction plays an overall protective or deleterious role in this type of injury response. To this end, our data cumulatively suggest that attenuating NF-κB induction following I/R injury in the liver results in substantially reduced inflammation and increased survival. Although these data do not eliminate the possibility that NF-κB plays a protective role in the liver following I/R, they do suggest that the overall level of NF-κB activation correlates with the detrimental induction of TNF-α and subsequent accompanying inflammatory properties. These data are consistent with previous studies demonstrating that reduced NF-κB activation afforded by ectopic expression of manganese superoxide dismutase is protective against liver I/R injury (47). From a therapeutic standpoint, the present study suggests that downregulating *IκBα* gene expression may be of potential therapeutic benefit in liver I/R injury.

Acknowledgments

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