IkBβ is an essential co-activator for LPS-induced IL-1β transcription in vivo

Melanie Scheibel, Bettina Klein, Heidrun Merkle, Manon Schulz, Ralph Fritsch, Florian R. Greten, Melek C. Arkan, Günter Schneider, and Roland M. Schmid

II. Medizinische Klinik, Technische Universität München, 81675 München, Germany

Inhibitor of κB (IkB) β (IkBβ) represents one of the major primary regulators of NF-κB in mammals. In contrast to the defined regulatory interplay between NF-κB and IkBα, much less is known about the biological function of IkBβ. To elucidate the physiological role of IkBβ in NF-κB signaling in vivo, we generated IkBβ-deficient mice. These animals proved to be highly refractory to LPS-induced lethality, accompanied by a strong reduction in sepsis-associated cytokine production. In response to LPS, IkBβ is recruited to the IL-1β promoter forming a complex with the NF-κB subunits RelA/c-Rel required for IL-1β transcription. Further transcriptome analysis of LPS-stimulated wild-type and IkBβ-deficient BM-derived macrophages revealed several other genes with known regulatory functions in innate immunity arguing that a subset of NF-κB target genes is under control of IkBβ. Collectively, these findings provide an essential proinflammatory role for IkBβ in vivo, and establish a critical function for IkBβ as a transcriptional coactivator under inflammatory conditions.

NF-κB plays an important role in the regulation of diverse biological processes such as development, immune and inflammatory responses, and apoptosis (Baldwin, 1996; Gilmore, 2006; Ghosh and Hayden, 2008). Through its ubiquitous appearance, NF-κB is involved in regulation of a wide range of genes, such as genes encoding cytokines, adhesion molecules, cytokine receptors, immunoregulatory molecules, and antiapoptotic proteins. In mammals, the NF-κB transcription factor family includes five members: p50/NF-κB1, p52/NF-κB2, RelA/p65, c-Rel, and RelB (Ghosh and Karin, 2002; Ghosh and Hayden, 2008). These polypeptide subunits form homo- and heterodimers that are sequestered through stable association with inhibitor of κB (IkB) proteins in the cytoplasm of resting cells. Activators of the NF-κB pathway, such as cytokines, growth factors, and bacterial and viral products, strongly enhance the activity of the IkB kinase complex (IKK). IKK phosphorylates the IkB inhibitor proteins, leading to their rapid proteasomal degradation (Karin and Ben-Neriah, 2000; Ghosh and Hayden, 2008). After degradation of IkB, NF-κB dimers are able to enter the nucleus, bind specifically to DNA, and modulate transcription of various target genes.

Because the initial discovery of the IkB proteins as the cytoplasmic inhibitors of NF-κB, considerable effort has been given to understand regulation and modes of action (Baeuerle and Baltimore, 1988). IkBα and IkBβ are the major signal-responsive isoforms within the IkB family that also includes IkBε, IkBγ, p100, p105, Bcl-3, and the newly described IkBζ (Yamamoto et al., 2004; Hoffmann and Baltimore, 2006). Although IkBα and IkBβ show many common structural features, they exhibit functional differences (Thompson et al., 1995; Tran et al., 1997). IkBα is rapidly degraded upon stimulation, followed by immediate NF-κB–dependent resynthesis. Newly synthesized IkBα enters the nucleus and removes NF-κB complexes from the DNA to export them back to the cytoplasm (Sun et al., 1993; Klement et al., 1996; Hoffmann et al., 2002). In contrast, IkBβ is degraded much more slowly, and its resynthesis is not regulated by NF-κB.
Depending on the cell type and stimulus, IkBβ undergoes persistent degradation, contributing to constitutive NF-κB activation (Thompson et al., 1995; Bourke et al., 2000). Furthermore, it has been shown that IkBα–NF-κB complexes undergo cytoplasmic to nuclear shuttling in resting cells, whereas IkBβ–NF-κB complexes commonly stay in the cytoplasm (Tran et al., 1997; Huang and Miyamoto, 2001; Malek et al., 2001; Ghosh and Karin, 2002). A hypophosphorylated form of IkBβ has been shown to reside in the nucleus of certain cell types upon stimulation. Nuclear IkBβ is capable of forming a complex with DNA–NF-κB dimers, but is unable to dislocate NF-κB from the DNA, thereby prolonging NF-κB activity (Suyang et al., 1996; DeLuca et al., 1999).

Accumulating evidence points to a broader nuclear function of the IkB protein family (Bates and Miyamoto, 2004). Certain IkB protein family members associate specifically with definite NF-κB proteins, acting as transcription coactivators at distinct genes. Thus, IkBα cooperates with RelA/p65 in the regulation of the Notch-target gene hes1 after stimulation with TNF (Aguilera et al., 2004). IkBβ associates specifically with p50 to the NF-κB-binding site of the IL-6 promoter (Yamamoto et al., 2004).

Less is known regarding the function of IkBβ in vivo. To analyze the physiological function of IkBβ, we generated IkBβ-deficient (IkBβ−/−) mice. We demonstrate that IkBβ−/− mice are highly resistant to LPS-induced septic shock. LPS resistance is caused by impaired cytokine expression in IkBβ−/− mice. Using the IL-1β gene as a model of IkBβ-regulated NF-κB target genes, we demonstrate that IkBβ is essential for IL-1β production upon LPS. In addition, we show that the transcription of IL-1β depends on a positively acting p65–c-Rel–IkBβ complex.

RESULTS
Generation and immunological phenotype of IkBβ−/− mice
To elucidate the physiological role of IkBβ, we generated IkBβ−/− mice using targeted gene disruption (as described in the Materials and methods section; Fig. 1 A). Exons 4 and 5 of the IkBβ gene, which code for the ankyrin repeats 4–6 that are essential for the function of IkB proteins and the binding to NF-κB, were deleted (Inoue et al., 1992). Southern blot analysis of the genomic tail DNA of F2 mice demonstrated a complete deletion of the IkBβ alleles (Fig. 1 B). Western blot analysis of whole spleen extracts indicated that IkBβ expression was completely abolished in IkBβ−/− mice with no change in the expression of IkBα and IkBε (Fig. 1 C).

IkBβ−/− mice born with the expected Mendelian frequency were viable and showed no distinct abnormalities in appearance. Flow cytometric analysis of isolated spleen cells from WT and IkBβ−/− mice demonstrated an increase in marginal zone B cells and a reduction of naive B cells (Fig. S1, A and B). Furthermore, increased memory T cell population in the spleen of IkBβ−/− mice was observed (Fig. S1 C) and analyses of BM exhibited an increase in BM-derived macrophages (BMDMs) in IkBβ−/− mice (Fig. S1 D).

IkBβ−/− mice are highly resistant to LPS-induced septic shock
Previous studies demonstrated that LPS stimulation leads to IkBβ degradation and persistent NF-κB activity. To elucidate the role of IkBβ during endotoxic shock, we analyzed the LPS responsiveness in IkBβ−/− mice (Thompson et al., 1995). Mice were intraperitoneally injected with a high dose of LPS (30 mg/kg), and survival was monitored (Fig. 2). IkBβ−/− mice demonstrate a remarkable resistance to the lethal effect of LPS (log-rank test IkBβ−/− versus WT, P < 0.0001) in contrast to WT animals that showed a 100% lethality within 36 h after LPS injection. Although surviving IkBβ−/− and IkBβ−/− mice showed signs of LPS-induced shock in the first hours, they completely recovered after 72 h, arguing for a significant protection against LPS-induced septic shock. The phenotype of the heterozygous mice suggests that both IkBβ alleles are required for the full LPS response in vivo.

Deficiency of IkBβ reduces the biosynthesis of the proinflammatory cytokines TNF, IL-1β, and IL-6
LPS induces rapid production of inflammatory cytokines in vivo, leading to multiorgan failure of the host (Morrison and Ryan, 1987). To determine whether increased resistance to endotoxic shock in IkBβ−/− mice was caused by decreased proinflammatory cytokines, we measured serum concentration of inflammatory cytokines TNF, IL-1β, and IL-6 after LPS challenge. As expected, the serum levels of these cytokines were significantly increased upon LPS treatment in WT mice (Fig. 3 A). In contrast, only a moderate increase of the serum concentrations of TNF, IL-1β, and IL-6 was observed in IkBβ−/− mice. Furthermore, mRNA levels of TNF, IL-1β, and IL-6 in the liver after LPS injection remained significantly lower in IkBβ−/− mice (Fig. 3 B). These data suggest that IkBβ is essential for in vivo production of inflammatory cytokines during LPS-induced septic shock.

IkBβ deficiency modify IL-1β cytokine production in LPS-stimulated BMDMs
As macrophages represent the major source of inflammatory cytokine production during sepsis, we analyzed cytokine expression in WT and IkBβ−/− BMDMs. In contrast to WT BMDMs that showed increased TNF, IL-6, and IL-1β mRNA expression and protein secretion upon LPS stimulation, endotoxin treatment led to impaired IL-1β mRNA induction, pro–IL-1β expression, and IL-1β secretion in IkBβ−/− BMDMs (Fig. 4, A and B). Although no significant changes in either TNF mRNA induction or secretion were observed after LPS treatment, IL-6 secretion was affected only at later time points in IkBβ−/− BMDMs (Fig. 4 A), suggesting specific regulation of IL-1β transcription by IkBβ in BMDMs. In addition to IL-1β, we observed in transcriptome profiles of LPS-stimulated WT and IkBβ−/− BMDMs that several other genes that are known to be important for the regulation of innate immunity are LPS induced in an IkBβ-dependent manner (Table S1).
to LPS, we stimulated cells with TNF and other TLR ligands. The \( \text{IkB} \) deficiency did not influence IL-1\( \beta \) secretion induced by TNF, CpgA (TLR9 agonist), and CpgB (TLR9 agonist) in BMDMs (Fig. S2). Although IL-1\( \beta \) secretion was decreased in polyI:C (TLR3 agonist) and Pam3CysSK4 (TLR2 agonist)-treated BMDMs, this reduction was not statistically significant (Fig. S2). Collectively, these data argue that \( \text{IkB} \) functions specifically in certain NF-\( \kappa \)B pathways.

### Influence of \( \text{IkB} \) small interfering RNA (siRNA) on IL-1\( \beta \) transcriptional regulation

To dissect the function of \( \text{IkB} \) in IL-1\( \beta \) expression in further detail in vitro, we used RNA interference in the macrophage cell line RAW264.7. Transfection of RAW264.7 macrophages with an \( \text{IkB} \)-specific siRNA led to a pronounced reduction in the \( \text{IkB} \) protein expression (Fig. 5 A). To test the \( \text{IkB} \) function in IL-1\( \beta \) secretion, we stimulated \( \text{IkB} \) siRNA-transfected RAW264.7 macrophages with LPS and measured IL-1\( \beta \) secretion over time (Fig. 5 B). Whereas control siRNA-transfected RAW264.7 cells secreted IL-1\( \beta \), IL-1\( \beta \) production was impaired in \( \text{IkB} \) siRNA-transfected cells (Fig. 5 B). In contrast, no change in LPS-induced secretion of other NF-\( \kappa \)B target genes MIP-2 and TNF were observed in \( \text{IkB} \) siRNA-transfected RAW264.7 macrophages, demonstrating specific interaction of \( \text{IkB} \) and IL-1\( \beta \) transcription (Fig. 5 B; Kim et al., 2003).

To further elucidate \( \text{IkB} \)-dependent regulation of the IL-1\( \beta \) promoter, we transfected several IL-1\( \beta \) reporter genes

### Figure 1. Disruption of the \( \text{IkB} \) gene.

(A) Schematic structure of WT \( \text{IkB} \) locus. Ankyrin repeats of \( \text{IkB} \) encoded by exons 2–5 are indicated. Furthermore, the targeting vector, the targeted \( \text{IkB} \) locus, the floxed \( \text{IkB} \) locus, and the \( \text{IkB} \) knock-out locus, generated by Cre recombination-mediated deletion of exons 4 and 5 are shown. Solid boxes represent exons, and lines represent introns. Neo, loxP-flanked PGK-neomycin cassette; HSV-tk, HSV-thymidine kinase gene; B, BamHI site. The length of BamHI-generated restriction fragments detected by Southern blotting with a 5' flanking probe is indicated. Location of the 5' flanking probe in exon 2 is shown. (B) Southern blot analysis of genomic DNA from targeted ES cells (+/3loxP), WT (+/+), and \( \text{IkB}^{-/-} \) F2 mice. (C) Immunoblot analysis of \( \text{IkB} \) in whole-cell extract of WT and \( \text{IkB}^{-/-} \) (ko) spleens. The membrane was stripped and probed for \( \beta \)-actin to ensure equal protein loading.
constructs into RAW264.7 macrophages (Fig. 5 C). The −518 bp IL-1β reporter gene, harboring two functional κB binding sites, showed an eightfold increase activity 8 h after stimulation with LPS in RAW264.7 macrophages (Cogswell et al., 1994; Fig. 5 D). Deleting the distal NF-κB-binding site (−399IL-1β) did not impair inducibility, whereas mutation or deletion of the proximal κB site significantly decreased LPS induction. These data suggest that the proximal κB site contributes to LPS-induction of the IL-1β promoter in RAW264.7 macrophages. However, we cannot completely exclude the contribution of the distal κB site.

To investigate whether IkBβ is essential for LPS-mediated induction of the IL-1β promoter, RAW267.6 macrophages were cotransfected with IkBβ siRNA and IL-1β reporter gene constructs. As shown in Fig. 5 E, knockdown of IkBβ clearly reduced IL-1β promoter induction after LPS stimulation, again pointing to a coactivator function of IkBβ toward IL-1β transcription.

Recruitment of IkBβ to the IL-1β promoter in complex with NF-κB p65–c-Rel

To test whether IkBβ directly binds to the IL-1β promoter, we performed chromatin immunoprecipitation (ChIP) assays. ChIP analysis using RAW264.7 macrophages demonstrated recruitment of IkBβ to the κB site of IL-1β promoter upon stimulation with LPS (Fig. 6 A). In contrast, IkBβ was not recruited to NF-κB–binding site in the MIP-2 gene promoter, suggesting specific regulation of the IL-1β promoter by IkBβ (Widmer et al., 1993). To characterize the activation complex in more detail, we investigated p65, p50, p52, and c-Rel binding. In addition to IkBβ, we found recruitment of the p65–c-Rel complex to the IL-1β promoter after LPS stimulation in RAW264.7 macrophages, suggesting that a p65–c-Rel dimer, assembled by IkBβ, induces IL-1β transcription after LPS treatment. In contrast, a classical NF-κB (p50/p65) dimer was recruited to the MIP-2 promoter upon LPS treatment and no binding of IkBβ was observed, demonstrating specificity for the IL-1β promoter (Fig. 6 A). Functionality of c-Rel for LPS-induced IL-1β transcription was also demonstrated using RNA interference in RAW264.7 macrophages (Fig. S3).

To further demonstrate the influence of IkBβ for dimer formation at the IL-1β promoter at the genetic level, we used ChIP assays in IkBβ−/− BMDMs. Binding of IkBβ to the IL-1β promoter upon LPS treatment was confirmed in BMDMs (Fig. 6 B). Whereas, c-Rel binds to the IL-1β promoter in WT macrophages, reduced binding was observed in IkBβ−/− BMDMs (Fig. 6 B). A significant lower binding of RNA polymerase II to the IL-1β promoter indicated decreased transcriptional activity in IkBβ−/− cells after LPS treatment (Fig. 6 B). On the other hand, complex formation and recruitment of RNA polymerase II after LPS treatment was not changed at the MIP-2 promoter gene in IkBβ−/− BMDMs, confirming specificity for the IL-1β promoter (Fig. 6 B).

Consistent with a nuclear function of IkBβ, we observed nuclear accumulation of IkBβ in LPS-treated BMDMs (Fig. S4). Previous studies demonstrated the interaction of IkBβ with c-Rel–p65 complexes in stimulated WEHI 231 cells in the nucleus (Phillips and Ghosh, 1997). To investigate direct interaction of the IkBβ–p65–c-Rel complex with DNA, we performed pulldown assays in Raw264.7 macrophages using biotinylated κB oligonucleotides corresponding to the proximal κB binding site of the IL-1β promoter. In nuclear extracts an IkBβ–p65–c-Rel trimer was found bound to the proximal κB binding site of the IL-1β promoter after LPS treatment (Fig. 6 C).

Figure 2. Survival of IkBβ−/− mice after high-dose LPS challenge.

Survival curves of IkBβ−/−, IkBβ+/−, and WT mice after the injection of LPS (30 mg/kg). Kaplan-Meier analysis demonstrated a significant difference in survival between IkBβ−/− and WT (log-rank test IkBβ−/− versus WT; P < 0.0001). Data are from three separate experiments and the number of mice in each group is indicated.

Figure 3. Deficiency in IkBβ reduces LPS–triggered production of sepsis inducing cytokines.

(A) Serum levels of IL-1β, TNF, and IL-6 in IkBβ−/− and control mice after LPS injection (30 mg/kg). IL-1β, and IL-6 were measured in serum collected from tail vein 0, 1, 2, and 6 h after injection of LPS (Student’s t test; *, P < 0.001 versus controls). (B) Liver IL-1β, TNF, and IL-6 mRNA expression levels after LPS challenge (30 mg/kg). At the indicated time points after LPS injection (30 mg/kg), total RNA from whole livers were prepared and mRNA levels were quantified using real-time PCR analysis (Student’s t test; *, P < 0.05 versus controls). For each time point, four animals per strain were examined in two independent experiments.
No binding of c-Rel was detected in unstimulated cells, indicating that LPS-signaling induces molecular changes, like p65–c-Rel dimer formation or conformational changes of c-Rel, which are needed to detect c-Rel binding to the κB oligonucleotide in the assay used. Altogether, these data suggest that IkBβ is recruited to the IL-1β promoter after LPS treatment and is needed for NF-κB complex formation and transcriptional activation.

**DISCUSSION**

Several reports of mice with targeted disruptions of IkB family members demonstrated that the different proteins play distinct biological roles. In contrast to IkBa deficient mice, much less is known regarding to the in vivo function of IkBβ. In this study, we analyzed IkBβ−/− mice and demonstrate a novel function of IkBβ in the whole organism. IkBβ−/− mice share none of the hallmarks compared with IkBa−/− mice (Beg et al., 1995; Klement et al., 1996). Similar to IkBa−/− mice, IkBβ−/− mice survive to adulthood and show no overt abnormalities (Mémet et al., 1999). However, our results demonstrate that IkBβ is essential in regulating innate immunity in a LPS model of septic shock.

Bacterial infection can induce a systemic response characterized by multiple organ failure and high mortality rate. LPS, a major integral structural component of the outer membrane of Gram-negative bacteria, is a potent initiator of inflammation and endotoxin shock. LPS activates macrophages to produce cytokines, such as IL-1β, TNF, and IL-6, which serve as critical mediators of septic shock (Morrison and Ryan, 1987). Excessive production of these cytokines leads to capillary leakage, vascular hemorrhage, tissue destruction, and subsequent lethal organ failure. Thus, the expression of proinflammatory cytokines such as IL-1β, TNF, and IL-6 needs to be tightly regulated during an inflammatory response. We now demonstrate that IkBβ is a critical regulator of LPS-induced septic shock. IkBβ deficiency confers LPS resistance in vivo, which is caused by the impaired secretion of the proinflammatory cytokines IL-1β, TNF, and IL-6. In BMDM, IL-1β was determined as a specific molecular IkBβ target, whereas the activation of the TNF and IL-6 genes remained unaffected after LPS treatment in this particular cellular model. Because IL-1β−/− mice are not protected from high-dose, LPS-induced septic shock, other IkBβ targets have to contribute to the observed LPS resistance (Fantuzzi et al., 1996). The importance of the IL-1 system for high-dose, LPS-induced septic shock is reflected by the LPS resistance of the IL-1β converting enzyme−deficient mice, known to have neither detectable serum levels of IL-1β nor IL-1α upon LPS challenge (Li et al., 1995). Interestingly, in microarray analysis of LPS-treated BMDMs, IL-1α induction after LPS stimulation was impaired in IkBβ−/− BMDMs (Table S1). However, Glaccum et al. (1997) reported that IL-1R−/− mice, which are refractory to both IL-1α and IL-1β signaling, are not resistant to LPS-induced septic shock, indicating that additional genes must contribute to the resistance in IkBβ−/− mice to LPS-induced lethality. The observation that several genes with important functions in innate immunity such as the chemokine (C-X-C motif) ligand 1 (Cxcl1), suppressor of cytokine signaling 3 (Socs3), interleukin 12p40 (Il12b) or others induced (C-X-C motif) ligand 1 (Cxcl1), suppressor of cytokine signaling 3 (Socs3), interleukin 12p40 (Il12b) or others induced...
Analysis of the IκBβ-dependent genes (Table S1) using Genomatix Pathway System software revealed a significant enrichment of genes controlled by the canonical IL-1–IKK–NF-κB signaling pathway (P < 0.01) and MyD88 response genes (P < 0.001), arguing that a subset of NF-κB– and MyD88-regulated genes is regulated by IκBβ (unpublished data). Furthermore, we observed a discrepancy between LPS-induced secretion of the proinflammatory cytokines in the BMDM model and LPS-induced cytokine expression measured in liver and serum. In BMDMs, only IL-1β secretion was impaired because of IκBβ deficiency, whereas the LPS-induced expression of IL-1β, TNF, and IL-6 was dependent on IκBβ in vivo. The liver is important for the initiation of defense mechanisms and the initiation of multiorgan failure during sepsis. LPS has been shown to activate hepatic Kupffer cells to synthesize and secrete inflammatory cytokines such as IL-1β, TNF, and IL-6 (Koo et al., 1999). Therefore, we cannot exclude that a different set of genes controlled by IκBβ in response to LPS in Kupffer cells, including IL-1β, TNF, and IL-6, are responsible for the LPS-resistance observed in IκBβ−/− mice in vivo. Thus, tissue and cell type specificities have to be considered in this context.

LPS is sensed by TLR4. Signaling via TLR4 activates a TRIF-dependent pathway of the induction of IFN-β and IFN-inducible genes in a MyD88-dependent pathway leading to activation of a NF-κB–dependent genetic program (Beutler, 2004; Beutler, 2009). The mechanisms by which LPS induces septic shock is related to its ability to activate NF-κB. For example, the highly LPS susceptible secretory leukoproteinase inhibitor–deficient mice are characterized by an increased NF-κB signaling magnitude, and the LPS-resistant poly ADP-ribose polymerase–deficient mice demonstrate a distinct impaired NF-κB activation (Oliver et al., 1999; Nakamura et al., 2003). IκBβ is thought to control late-phase NF-κB activation (Hoffmann et al., 2002). This IκBβ activity was not observed in LPS-stimulated BMDMs because LPS-stimulation results in similar NF-κB activation kinetics in WT and IκBβ−/− mice. Because IκBe is present in IκBβ−/− cells and degraded with the same kinetics as in WT BMDMs, IκBe may compensate for the IκBβ loss.

We detected a gene-specific activator function of IκBβ during the early LPS-induced NF-κB response. As a model of IκBβ–dependent transcriptional regulation, we focused onto the control of the IL-1β promoter because induction of this gene by LPS mostly depends on IκBβ, revealing a tenfold decreased inducibility in WT compared with IκBβ−/− BMDMs (Table S1). We observed a direct recruitment of IκBβ to the κB-binding site of the IL-1β promoter in complex with p65/c-Rel. Interestingly, in BMDMs of IκBβ−/− mice the lack of IκBβ binding to the IL-1β promoter leads to the loss of c-Rel recruitment as well as reduced binding of RNA polymerase II, indicating reduced transcriptional...
activation. Therefore, the remaining p65/RelA homodimers are not sufficient to activate IL-1β transcription, suggesting that IkBβ is indispensable in formation of a transcriptional active p65–c-Rel complex at the IL-1β promoter. This is in line with recent observations, demonstrating that each NF-κB dimer supports a different amount of transcriptional activation at a specific gene promoter and that the IL-1β gene-promoter is most responsive to p65/RelA and c-Rel in vitro (Algarté et al., 1999; Hiscott et al., 1993; Lin et al., 1995; Saccani et al., 2003).

As previously characterized, a stable complex of IkBβ and NF-κB p65/c-Rel complex in the nucleus of RAW264.7 macrophages was not shown to be NF-κB p50–p65–IkBβ of LPS-induced genes, like IL-6, by forming a promoter-bound transcription factor. We suggest a more active role for IkBβ in gene transcription than a sole chaperone function. Because IkBβ is clearly needed to recruit c-Rel to the IL-1β gene promoter, we suggest a more active role for IkBβ in gene transcription than a sole chaperone function.

In addition to the inhibitory function of the IkB protein family in resting cells, promoter-specific functions are becoming more evident. LPS signaling induces expression of IkB family in resting cells, promoter-specific functions are becoming more evident. LPS signaling induces expression of IkB in macrophages, which is important for the induction of a subset of LPS-induced genes, like IL-6, by forming a promoter-bound p50–p65–IkB complex. Interestingly, LPS induction of IL-6 in macrophages was not shown to be IkBβ dependent (Yamamoto et al., 2004). Together with our data, which demonstrates specificity of IkBβ toward certain NF-κB– and MyD88-regulated promoters and recruitment of a p65/c-Rel dimer to the IL-1β promoter, we propose that the IkBβ proteins function to confer selectivity in NF-κB dimer usage, and therefore in signaling specificity. In addition to IkBβ and IkBα, IkBα was shown to repress the hes1 promoter by direct binding (Aguilera et al., 2004). Furthermore, it was demonstrated that IkBα interacts with corepressors, like SMRT and N-CoR and different histone deacetylases (Aguilera et al., 2004). Whether IkBβ interacts with the epigenetic machinery is unknown in the moment and awaits further experiments.

Together, we now provide genetic evidence that IkBβ is essential for resistance toward LPS induced septic-shock. At the molecular level, IkBβ binds to a subset of NF-κB-dependent promoters and activates a subset of LPS-induced genes, like the IL-1β gene. This establishes IkBβ as an essential coactivator for gene transcription in vivo.

**MATERIALS AND METHODS**

**Targeted disruption of the IkBβ gene.** The 8.2-kb genomic clone containing exons 3–6 of IkBβ was isolated from a genomic 129/Sv JEM VOL. 207, November 22, 2010

Article

Figure 6. Recruitment of IkBβ to the IL-1β promoter in complex with NF-κB p65/c-Rel. (A) ChIP in RAW264.7 macrophages. RAW264.7 macrophages were stimulated for 2 h with LPS (100 ng/ml). Chromatin was immunoprecipitated with IkBβ, RelA/p65, NF-κB1/p50, NF-κB2/p52, c-Rel, and RNA-polymerase II–specific antibodies or IgG as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene-specific primers for IL-1β or MIP-2 promoters. Three independent experiments revealed similar results. (B) ChIP in BMDMs. After stimulation with LPS (100 ng/ml) for 1 h, chromatin was immunoprecipitated with IkBβ, RelA/p65, NF-κB1/p50, NF-κB2/p52, c-Rel, and RNA-polymerase II–specific antibodies or IgG as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene-specific primers for IL-1β or MIP-2 promoters. Three independent experiments revealed similar results. (C) Biotin-streptavidin pulldown assay with a kβ oligonucleotide, corresponding to the proximal kβ site of the IL-1β promoter. RAW264.7 macrophages were stimulated with LPS (100 ng/ml) for 2 h. Nuclear and cytoplasmic extracts were incubated with the kβ oligonucleotide and pulled down with streptavidin-agarose. Western blot detected RelA/p65, c-Rel, and IkBβ. One out of three independent experiments is shown.
clones were analyzed by Southern blot and PCR analyses to validate the correct deletion of the neo-cassette. These clones were aggregated to C57BL/6 mor-
ulak, and resulting chimeric mice were crossed with Dleter-Cre-mice to generate IkBβ knockout mice (Schwenk et al., 1995). Disruption of the IkBβ gene was verified by Southern blot and PCR analyses of tail DNA. Homozygous offspring were observed at the predicted frequency by interbreeding hetero-
ygous mice. The null phenotype created by mutation of the IkBβ gene was confirmed by Western blot analysis of spleen extracts. For LPS injection, IkBβ−/− mice were backcrossed at least 7 times to the C57BL/6 background.

Reagents. CpG 1826 B-type, CpG 2216 A-type, PAM3CSK4, and PolyIC were purchased from Sigma-Aldrich.

Systemic challenge of WT and IkBβ−/− mice. WT C57BL/6 (litter-
ate controls or purchased from Charles River Laboratories), IkBβ+/− litter-
ters, and IkBβ−/− mice were injected i.p. with LPS 30 mg/kg (L-2630, strain 0111:B4; Sigma-Aldrich). Mice were monitored over 72 h for signs of sepsis and lethality. Blood was taken from the tail vein 0, 1, 2, and 6 h after LPS injection to investigate serum levels of IL-1β, TNF, and IL-6 using ELISA. All animal studies were conducted in compliance with European guidelines for the care and use of laboratory animals and were approved by the local authorities (Regierung von Oberbayern).

Immunocytochemistry. For immunodetection of IkBβ and RelA/p65 in WT and IkBβ−/− LPS or untreated BMDMs, cells were fixed in 4% formal-
dehyde (Sigma-Aldrich), permeabilized with 0.3% Triton-X (Sigma-
Aldrich), followed by a FITC-labeled secondary anti-
body (Invitrogen). Cells were then counterstained with DAPI (Vector Laboratories) to identify nuclei and subjected to fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Inc.). Emitted fluorescence was collected on a color charge-coupled device camera (AxioCam MRc; Carl Zeiss, Inc.). High-resolution images were captured and analyzed using AxioVision 4.3 software (Carl Zeiss, Inc.).

ChIP. ChIP analyses were performed as previously described (Fritsche et al., 2009; Schneider et al., 2006, 2010). An equal amount of total RNA was isolated from liver, BMDMs, or RAW264.7 macrophages was isolated using the RNeasy kit (Qiagen) following the manufacturer’s instructions. Quantitative mRNA analyses were performed as previously described for real-time PCR analy-
Sd (TaqMan, PE Applied Biosystems; Schneider et al., 2006). The following antibodies were used: IkBβ-β, RelA/p65, c-Rel, p50, p52, RNA-Polymerase II, and control IgG, all from Santa Cruz Biotechnology, Inc., followed by a FITC-labeled secondary anti-
body (Invitrogen). Cells were then counterstained with DAPI (Vector Laboratories) to identify nuclei and subjected to fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Inc.). Emitted fluorescence was collected on a color charge-coupled device camera (AxioCam MRc; Carl Zeiss, Inc.). High-resolution images were captured and analyzed using AxioVision 4.3 software (Carl Zeiss, Inc.).

Quantitative real-time RT-PCR. Total RNA was isolated from liver, BMDMs, or RAW264.7 macrophages was isolated using the RNasey kit (Qiagen) following the manufacturer’s instructions. Quantitative mRNA analyses were performed as previously described for real-time PCR analy-
sis (TaqMan, PE Applied Biosystems; Schneider et al., 2006). The following antibodies were used: IkBβ-β, RelA/p65, c-Rel, p50, p52, RNA-Polymerase II, and control IgG, all from Santa Cruz Biotechnology, Inc., followed by a FITC-labeled secondary anti-
body (Invitrogen). Cells were then counterstained with DAPI (Vector Laboratories) to identify nuclei and subjected to fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Inc.). Emitted fluorescence was collected on a color charge-coupled device camera (AxioCam MRc; Carl Zeiss, Inc.). High-resolution images were captured and analyzed using AxioVision 4.3 software (Carl Zeiss, Inc.).

Flow cytometry. Fluorescence staining of isolated mouse splenocytes was performed as described previously (Rad et al., 2006). The following antibodies were used: PE-conjugated anti-IgD (SouthernBiotech); biotinylated anti-
CD3 (Caltag Laboratories); PE-conjugated anti-Terr-119, PE-conjugated anti-CD45RB, PE-conjugated anti-CD19, APC-conjugated anti-CD62L, FITC-conjugated anti-CD23, FITC-conjugated anti-CD21, biotinyl-
ted anti-IgM, APC-conjugated anti-CD11b, biotinylated anti-CD3 (BD). Streptavidin-PerCP was from BD Fluorescence was analyzed by using a FACScalibur (BD) flow cytometer and CellQuest software (BD).

Biotin-streptavidin pulldown assay. Assays were performed as previously described (Schild et al., 2009, Schneider et al., 2010). Approximately 7 × 10^7 RAW264.7 macrophages were used for each time point. Nuclear and cyto-
solic extract were prepared by using nuclear extraction kit (Active Motif) according to the manufacturer’s instructions. The following 5’ biotin-labeled oligonucleotide, corresponding to the positions +262 to +270 of the IL-1β promoter, was used: 5’-AACCCCGAAAAACCAATATT-T3’. Flow cytometry. Fluorescence staining of isolated mouse splenocytes was performed as described previously (Rad et al., 2006). The following antibodies were used: PE-conjugated anti-IgD (SouthernBiotech); biotinylated anti-
CD3 (Caltag Laboratories); PE-conjugated anti-Terr-119, PE-conjugated anti-CD45RB, PE-conjugated anti-CD19, APC-conjugated anti-CD62L, FITC-conjugated anti-CD23, FITC-conjugated anti-CD21, biotinyl-
ted anti-IgM, APC-conjugated anti-CD11b, biotinylated anti-CD3 (BD). Streptavidin-PerCP was from BD Fluorescence was analyzed by using a FACScalibur (BD) flow cytometer and CellQuest software (BD).

11B+ promoter reporter gene assay and mutagenesis. To determine 11B+ promoter activity the −518–11B-luc, −399–11B-luc, and −199–
11B-luc luciferase reporter gene constructs were used. Point mutations within the −399–11B-luc plasmid were generated using the QuikChange Site-Directed Mutagenesis kit (Strategies) in conjunction with the following oligonucleotides: 5’-CTTCTTTCTACGAGTTAGTTAGAATCCATTTATTTGTGAC-3’ and 5’-GCACCTAATTGAAATGCAAATCACTAGAA-3’. Mutated residues are shown in Fig. 5 C. Mutated probes were generated by sequencing. 1 µg of each reporter gene construct was transfected using Oligofectamine (Invitrogen). Luciferase activity was normalized to protein concentration and analyzed as previously described (Reichert et al., 2007).

Assay with siRNA specific to IkBβ and c-Rel. RAW264.7 macro-
phages were transfected with siRNA duplex (Ambion) specific for mouse IkBβ or with scramble duplex in a concentration of 200 nM with Oligo-
fectamine (Invitrogen) according to the manufacturer’s instructions. The fol-
lowing siRNAs were used: IkBβ, 5’-GACUGAGGGCUCAACAUAG-3’; c-Rel, 5’-UAAGCAUGUGUUGACAGAUACU-3’; control siRNA, 5’-CAGUCGGUUGUUCGACUG-3’. EMSAs. EMSAs were performed as previously described (Arkan et al., 2001) using NF-κB 5’-AGTGTAGGGGACTTCTCCAGGC-3’ and 3’-TCACTCCCTGAAAGGGTCCG-5’ and Oct-1 5’-TCTGAG-
TACTACACAACTAGA-3’ and 3’-ACACGTTCAGTTGTAGTGA-
TCTT-5’) oligonucleotides.
Gene expression profiling. Gene expression profiling was performed as previously described (Retchert et al., 2007; Frutsche et al., 2009). Duplexes of total RNA were prepared using RNeasy kit (Qiagen). Labeled cRNA was produced and hybridized onto the Affymetrix GeneChip Mouse Genome 430 2.0 Array set according to Affymetrix standard protocols. Expression data were analyzed using Microarray Suite 5.0. Genes induced at least fivefold in WT BMDMs 2 h after LPS stimulation (100 ng/ml) and whose induction is reduce to <5% in IκBβ−/− compared with WT BMDMs are presented in Table S1.

Statistical analysis. Unless otherwise indicated, all data were obtained from at least three independent experiments performed in triplicate and the results are presented as mean and standard error of the mean (SEM). To demonstrate statistical significance a two-tailed Student’s t test or Kaplan-Meier with a log-rank test was used. Statistical significance was assigned to P < 0.05.

Online supplemental material. Fig. S1 shows that IκBβ mice−/− demonstrate an increase of splenic marginal zone B cells and memory T cells and an enforced differentiation of macrophages within the BM. Fig. S2 shows on demonstrate an increase of splenic marginal zone B cells and memory T cells and a noncon- sensus CRE-like site. J. Immunol. 153:712–723.

Deluca, C., L. Petropoulos, D. Zeneuareu, and J. Hiscott. 1999. Nuclear IκappaBβ maintains persistent NF-κappaB activation in HIV-1-infected myeloid cells. J. Biol. Chem. 274:13010–13016. doi:10.1074/jbc.274.19.13010

Fantuzzi, G., H. Zheng, R. Faggioni, F. Benigni, P. Ghezzi, J.D. Sipe, A.R. Shaw, and C.A. Dinarello. 1996. Effect of endotoxin in IL-1 beta-deficient mice. J. Immunol. 157:291–296.

Frunspe, P., B. Seidler, S. Schuler, A. Schnekue, M. Gottlick, R.M. Schadb, D. Saur, and G. Schneider. 2009. HDAC2 mediates therapeu- stic resistance of pancreatic cancer cells via the BH3-only protein NOXA. Gastroenterology. 58:1399–1409. doi:10.11136/gut.2009.180711

Ghis, S., M.S. Hayden. 2008. New regulators of NF-κappaB in inflam- mation. Nat. Rev. Immunol. 8:837–848. doi:10.1038/nri2423

Ghis, S., and M. Karon. 2002. Musing pieces in the NF-κappaB puzzle. Cell. 109(Suppl):S81–S96. doi:10.1016/S0092-8674(02)00703-1

Ginobre, T.D. 2006. Introduction to NF-κappaB: players, pathways, perspectives. Oncogene. 25:6680–6684. doi:10.1080/0261537050099594

Glaccmb, M.C., K.L. Stocking, K. Carthier, J.L. Smith, C.R. Willis, C. Maliszewski, D.J. Livingston, J.J. Peshon, and P.J. Morrissey. 1997. Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. J. Immunol. 159:3364–3371.

Hiscox, J., J. Marosis, J. Garoufalis, M. D’Addario, A. Roulston, I. Kwon, N. Pepin, J. Lacoste, H. Nguyen, G. Beni, et al. 1993. Characterization of a functional NF-κappaB site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. Mol. Cell. Biol. 13:6231–6240.

Hoffmann, A., and D. Baltimore. 2006. Circuitry of nuclear factor kappaB signaling. Immunity. 20:171–186. doi:10.1111/j.0105-2896.2006.00375.x

Hoffmann, A., A. Levencheno, M.L. Scott, and D. Baltimore. 2002. The IkappaB-NF-κappaB signaling module: temporal control and selective gene activation. Science. 298:1241–1245. doi:10.1126/science.1071914

Huang, T.T., and S. Miyamoto. 2001. Postexpression activation of NF-κappaB requires the amino-terminal nuclear export signal specific to IkappaBalpha. Mol. Cell. Biol. 21:4737–4747. doi:10.1128/MCB.21.14.4737–4747.2001

Inoue, J., L.D. Kerr, D. Rashid, N. Davis, H.R. Bose Jr., and I.M. Verma. 1992. Direct association of pp40/IKappa B beta with rel/NF-κappaB B transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA. 89:4333–4337. doi:10.1073/pnas.89.10.4333

Karim, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappaB]-beta activity. Annu. Rev. Immunol. 18:621–663. doi:10.1146/annurev.immunol.18.1.621

Kim, D.S., J.H. Han, and H.J. Kwon. 2003. NF-κappaB and c-Jun-dependent regulation of macrophage inflammatory protein-2 gene expression in response to lipopolysaccharide in RAW 264.7 cells. Mol. Immunol. 40:633–643. doi:10.1016/j.molimm.2003.07.001

Klement, J.F., N.R. Rice, B.D. Car, S.J. Abbondanzo, G.D. Powers, P.H. Bhatt, C.H. Chen, C.A. Rosen, and C.L. Stewart. 1996. IkappaBalpha deficiency results in a sustained NF-kappaB response and severe wide- spread dermatitis in mice. Mol. Cell. Biol. 16:2341–2349. doi:10.1128/mcb.16.4.2341-2349.1996

Koo, D.J., I.H. Chaudry, and P. Wang. 1999. Kupffer cells are responsible for producing inflammatory cytokines and hepatocellular dysfunction during early sepsis. J. Surg. Res. 83:151–157. doi:10.1006/jscr.1999.5584

Bi, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, et al. 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell. 80:401–411. doi:10.1016/0092-8674(95)00490-5

Lin, R., D. Gewert, and J. Hiscott. 1995. Differential transcriptional activation in vitro by NF-kappaB B/Rel proteins. J. Biol. Chem. 270:3125–3131. doi:10.1074/jbc.270.7.3123

Malek, S., Y. Chen, T. Husford, and G. Ghosh. 2001. IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization.
sequences in resting cells. J. Biol. Chem. 276:45225–45235. doi:10.1074/jbc.M105865200

Ménet, S., D. Laouini, J.C. Epinat, S.T. Whiteside, B. Goudeau, D. Philpott, S. Kayal, P.J. Sansonetti, P. Berche, J. Kanellopoulos, and A. Israel. 1999. IκBε-deficient mice: reduction of one T cell precursor subspecies and enhanced Ig isotype switching and cytokine synthesis. J. Immunol. 163:5994–6005.

Morrison, D.C., and J.L. Ryan. 1987. Endotoxins and disease mechanisms. Annu. Rev. Med. 38:417–432.

Nakamura, A., Y. Mori, K. Hagiwara, T. Suzuki, T. Sakakibara, T. Kikuchi, T. Igarashi, M. Ebina, T. Abe, J. Miyazaki, et al. 2003. Increased susceptibility to LPS-induced endotoxin shock in secretory leukoprotease inhibitor (SLPI)-deficient mice. J. Exp. Med. 197:669–674. doi:10.1084/jem.20021824

Ohashi, K., V. Burkart, S. Flohé, and H. Kolb. 2000. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J. Immunol. 164:558–561.

Oliver, F.J., J. Ménissier-de Murcia, C. Nacci, P. Decker, R. Andriantsitohaina, S. Muller, G. de la Rubia, J.C. Stoclet, and G. de Murcia. 1999. Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly (ADP-ribose) polymerase-1 deficient mice. EMBO J. 18:4446–4454. doi:10.1093/emboj/18.16.4446

Phillips, R.J., and S. Ghosh. 1997. Regulation of IkappaB beta in WEHI 231 mature B cells. Mol. Cell. Biol. 17:4390–4396.

Rad, R., L. Brenner, S. Bauer, S. Schwendy, L. Layland, C.P. da Costa, W. Reindl, A. Dossoumbekova, M. Friedrich, D. Saur, et al. 2006. CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. Gastroenterology. 131:525–537. doi:10.1053/j.gastro.2006.05.001

Reichert, M., D. Saur, R. Hamacher, R.M. Schmid, and G. Schneider. 2007. Phosphoinositide-3-kinase signaling controls S-phase kinase-associated protein 2 transcription via E2F1 in pancreatic ductal adenocarcinoma cells. Cancer Res. 67:4149–4156. doi:10.1158/0008-5472.CAN-06-4484

Saccani, S., S. Pantano, and G. Natoli. 2003. Modulation of NF-κB activity by exchange of dimers. Mol. Cell. 11:1563–1574. doi:10.1016/S1097-2765(03)00227-2

Schild, C., M. Wirth, M. Reichert, R.M. Schmid, D. Saur, and G. Schneider. 2009. PLK signaling maintains c-myc expression to regulate transcription of E2F1 in pancreatic cancer cells. Mol. Carcinog. 48:1149–1158. doi:10.1002/mc.20569

Schneider, G., D. Saur, J.T. Siveke, R. Fritsch, F.R. Greten, and R.M. Schmid. 2006. IKKalpha controls p52/RelB at the skp2 gene promoter to regulate G1- to S-phase progression. EMBO J. 25:3801–3812. doi:10.1038/sj.emboj.7601259

Schneider, G., A. Henrich, G. Greiner, V. Wolf, A. Lovas, M. Wieczorek, T. Wagner, S. Reichardt, A. von Werder, R.M. Schmid, et al. 2010. Cross talk between stimulated NF-κBalpha and the tumor suppressor p53. Oncogene. 29:2795–2806. doi:10.1038/onc.2010.46

Schwenk, F., U. Baron, and K. Rajewsky. 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23:5080–5081. doi:10.1093/nar/23.24.5080

Sun, S.C., P.A. Ganchi, D.W. Ballard, and W.C. Greene. 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science. 259:1912–1915. doi:10.1126/science.8096691

Suyang, H., R. Phillips, I. Douglas, and S. Ghosh. 1996. Role of unphosphorylated, newly synthesized I kappa B alpha in persistent activation of NF-κB. Mol. Cell. Biol. 16:5444–5449.

Thompson, J.E., R.J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh. 1995. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. Cell. 80:573–582. doi:10.1016/0092-8674(95)90511-1

Tran, K., M. Merika, and D. Thanos. 1997. Distinct functional properties of IkappaB alpha and IkappaB beta. Mol. Cell. Biol. 17:5386–5399.