

I κ B β is an essential co-activator for LPS-induced IL-1 β transcription in vivo

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Inhibitor of κ B (I κ B) β (I κ B β) represents one of the major primary regulators of NF- κ B in mammals. In contrast to the defined regulatory interplay between NF- κ B and I κ B α , much less is known about the biological function of I κ B β . To elucidate the physiological role of I κ B β in NF- κ B signaling in vivo, we generated I κ B β -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, I κ B β is recruited to the IL-1 β promoter forming a complex with the NF- κ B subunits RelA/c-Rel required for IL-1 β transcription. Further transcriptome analysis of LPS-stimulated wild-type and I κ B β -deficient BM-derived macrophages revealed several other genes with known regulatory functions in innate immunity arguing that a subset of NF- κ B target genes is under control of I κ B β . Collectively, these findings provide an essential proinflammatory role for I κ B β in vivo, and establish a critical function for I κ B β as a transcriptional coactivator under inflammatory conditions.

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Abbreviations used: BMDM, BM-derived macrophage; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; I κ B, inhibitor of κ B; IKK, I κ B kinase; siRNA, small interfering RNA; TLR, Toll-like receptor.

NF- κ B 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- κ B 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- κ B transcription factor family includes five members: p50/NF- κ B1, p52/NF- κ B2, 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 κ B (I κ B) proteins in the cytoplasm of resting cells. Activators of the NF- κ B pathway, such as cytokines, growth factors, and bacterial and viral products, strongly enhance the activity of the I κ B kinase complex (IKK). IKK phosphorylates the I κ B inhibitor proteins, leading to their rapid proteasomal degradation (Karin and Ben-Neriah, 2000; Ghosh and Hayden, 2008). After degradation of I κ B, NF- κ B dimers are able to enter

the nucleus, bind specifically to DNA, and modulate transcription of various target genes.

Because the initial discovery of the I κ B proteins as the cytoplasmic inhibitors of NF- κ B, considerable effort has been given to understand regulation and modes of action (Baeuerle and Baltimore, 1988). I κ B α and I κ B β are the major signal-responsive isoforms within the I κ B family that also includes I κ B ϵ , I κ B γ , p100, p105, Bcl-3, and the newly described I κ B ζ (Yamamoto et al., 2004; Hoffmann and Baltimore, 2006). Although I κ B α and I κ B β show many common structural features, they exhibit functional differences (Thompson et al., 1995; Tran et al., 1997). I κ B α is rapidly degraded upon stimulation, followed by immediate NF- κ B-dependent resynthesis. Newly synthesized I κ B α enters the nucleus and removes NF- κ B 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, I κ B β is degraded much more slowly, and its resynthesis is not regulated by NF- κ B.

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Depending on the cell type and stimulus, I κ B β undergoes persistent degradation, contributing to constitutive NF- κ B activation (Thompson et al., 1995; Bourke et al., 2000). Furthermore, it has been shown that I κ B α -NF- κ B complexes undergo cytoplasmic to nuclear shuttling in resting cells, whereas I κ B β -NF- κ B 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 I κ B β has been shown to reside in the nucleus of certain cell types upon stimulation. Nuclear I κ B β is capable of forming a complex with DNA-NF- κ B dimers, but is unable to dislocate NF- κ B from the DNA, thereby prolonging NF- κ B activity (Suyang et al., 1996; DeLuca et al., 1999).

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

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

RESULTS

Generation and immunological phenotype of I κ B β ^{-/-} mice

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

I κ B β ^{-/-} 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 I κ B β ^{-/-} 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 I κ B β ^{-/-} mice was observed (Fig. S1 C) and analyses of BM exhibited an increase in BM-derived macrophages (BMDMs) in I κ B β ^{-/-} mice (Fig. S1 D).

I κ B β ^{-/-} mice are highly resistant to LPS-induced septic shock

Previous studies demonstrated that LPS stimulation leads to I κ B β degradation and persistent NF- κ B activity. To elucidate the role of I κ B β during endotoxic shock, we analyzed the LPS responsiveness in I κ B β ^{-/-} mice (Thompson et al., 1995). Mice were intraperitoneally injected with a high dose of LPS (30 mg/kg), and survival was monitored (Fig. 2). I κ B β ^{-/-} mice demonstrate a remarkable resistance to the lethal effect of LPS (log-rank test I κ B β ^{-/-} versus WT, $P < 0.0001$) in contrast to WT animals that showed a 100% lethality within 36 h after LPS injection. Although surviving I κ B β ^{+/-} and I κ B β ^{-/-} 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 I κ B β alleles are required for the full LPS response in vivo.

Deficiency of I κ B β reduces the biosynthesis of the proinflammatory cytokines TNF, IL-1 β , 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 I κ B β ^{-/-} mice was caused by decreased proinflammatory cytokines, we measured serum concentration of inflammatory cytokines TNF, IL-1 β , 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-1 β , and IL-6 was observed in I κ B β ^{-/-} mice. Furthermore, mRNA levels of TNF, IL-1 β , and IL-6 in the liver after LPS injection remained significantly lower in I κ B β ^{-/-} mice (Fig. 3 B). These data suggest that I κ B β is essential for in vivo production of inflammatory cytokines during LPS-induced septic shock.

I κ B β deficiency modify IL-1 β 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 I κ B β ^{-/-} BMDMs. In contrast to WT BMDMs that showed increased TNF, IL-6, and IL-1 β mRNA expression and protein secretion upon LPS stimulation, endotoxin treatment led to impaired IL-1 β mRNA induction, pro-IL-1 β expression, and IL-1 β secretion in I κ B β ^{-/-} 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 I κ B β ^{-/-} BMDMs (Fig. 4 A), suggesting specific regulation of IL-1 β transcription by I κ B β in BMDMs. In addition to IL-1 β , we observed in transcriptome profiles of LPS-stimulated WT and I κ B β ^{-/-} BMDMs that several other genes that are known to be important for the regulation of innate immunity are LPS induced in an I κ B β -dependent manner (Table S1).

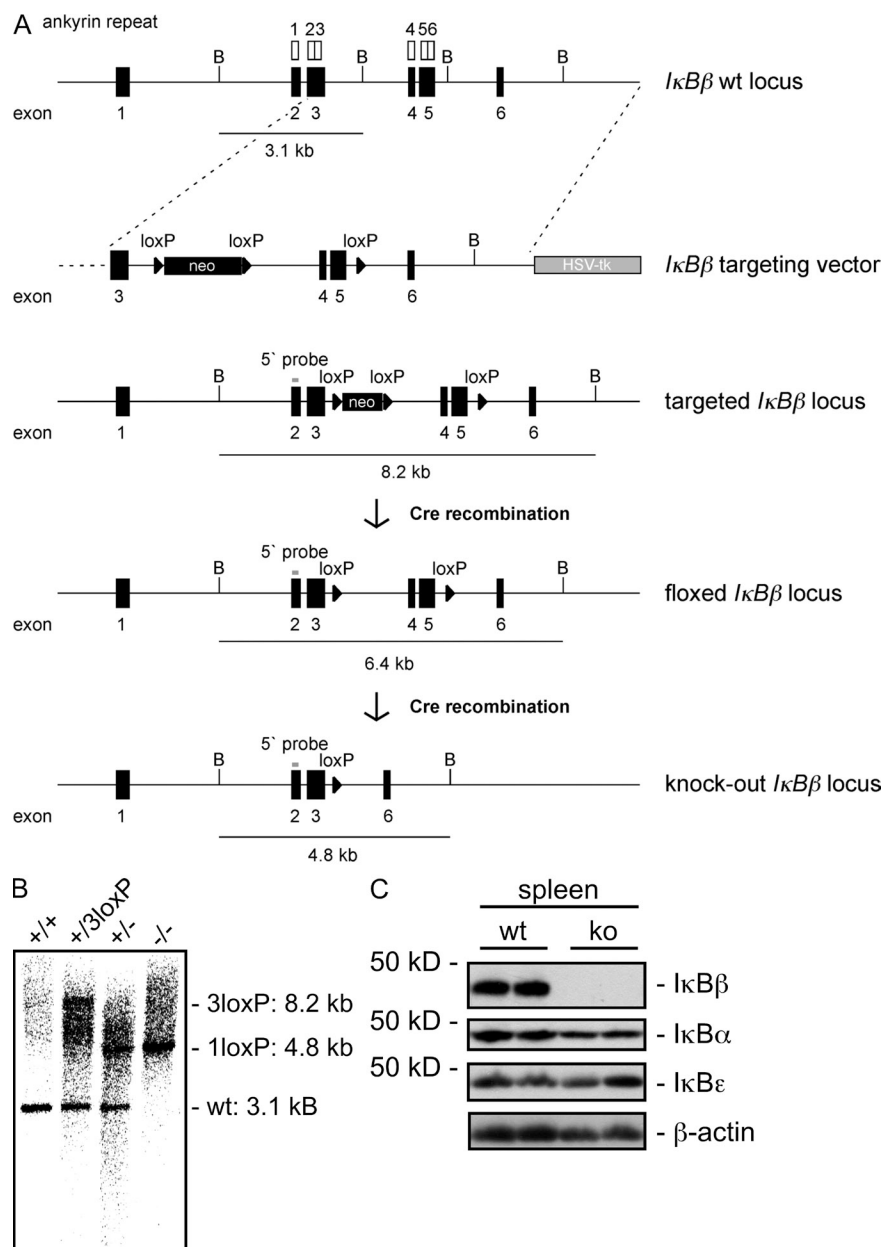


Figure 1. Disruption of the *IκBβ* gene.

(A) Schematic structure of WT *IκBβ* locus. Ankyrin repeats of *IκBβ* encoded by exons 2–5 are indicated. Furthermore, the targeting vector, the targeted *IκBβ* locus, the floxed *IκBβ* locus, and the *IκBβ* 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 (+/+) mice, *IκBβ*^{+/-}, and *IκBβ*^{-/-} F₂ mice. (C) Immunoblot analysis of *IκBβ*, *IκBα*, and *IκBε* in whole-cell extract of WT and *IκBβ*^{-/-} (ko) spleens. The membrane was stripped and probed for β-actin to ensure equal protein loading.

to LPS, we stimulated cells with TNF and other TLR ligands. The *IκBβ* deficiency did not influence IL-1β secretion induced by TNF, CpgA (TLR9 agonist), and CpgB (TLR9 agonist) in BMDMs (Fig. S2). Although IL-1β 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 *IκBβ* functions specifically in certain NF-κB pathways.

Influence of *IκBβ* small interfering RNA (siRNA) on IL-1β transcriptional regulation

To dissect the function of *IκBβ* in IL-1β expression in further detail in vitro, we used RNA interference in the macrophage cell line RAW264.7. Transfection

of RAW264.7 macrophages with an *IκBβ*-specific siRNA led to a pronounced reduction in the *IκBβ* protein expression (Fig. 5 A). To test the *IκBβ* function in IL-1β secretion, we stimulated *IκBβ* siRNA-transfected RAW264.7 macrophages with LPS and measured IL-1β secretion over time (Fig. 5 B). Whereas control siRNA-transfected RAW264.7 cells secreted IL-1β, IL-1β production was impaired in *IκBβ* siRNA-transfected cells (Fig. 5 B). In contrast, no change in LPS-induced secretion of other NF-κB target genes MIP-2 and TNF were observed in *IκBβ* siRNA-transfected RAW264.7 macrophages, demonstrating specific interaction of *IκBβ* and IL-1β transcription (Fig. 5 B; Kim et al., 2003).

To further elucidate *IκBβ*-dependent regulation of the IL-1β promoter, we transfected several IL-1β reporter gene

Binding of LPS to Toll-like receptor 4 (TLR4) activates NF-κB through IKK in a MyD88-dependent manner. To test for altered signaling kinetics and DNA binding in *IκBβ*^{-/-} BMDMs, we performed electrophoretic mobility shift assays (EMSA). Once normalized to Oct1 DNA binding, LPS-induced NF-κB signaling kinetics were indistinguishable (Fig. 4 C). No differences in the protein expression level of NF-κB1 or NF-κB2 were evident in BMDMs of *IκBβ*^{-/-} mice (unpublished data). RelA/p65 expression was decreased and c-Rel expression increased in BMDMs of *IκBβ*^{-/-} mice (Fig. 4 D). *IκBα* was phosphorylated and both, *IκBα* and *IκBε*, were degraded with the same kinetics upon LPS treatment in WT and *IκBβ*^{-/-} mice (Fig. 4 D). To determine whether the defect of *IκBβ*^{-/-} macrophages that produce IL-1β was specific

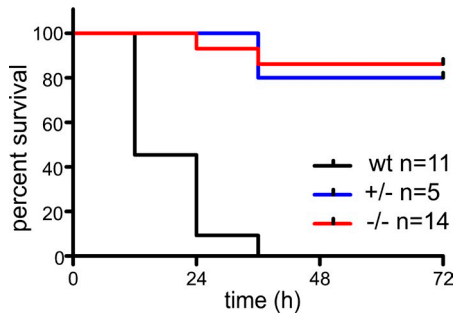


Figure 2. Survival of $\text{I}\kappa\text{B}\beta^{-/-}$ mice after high-dose LPS challenge. Survival curves of $\text{I}\kappa\text{B}\beta^{-/-}$, $\text{I}\kappa\text{B}\beta^{+/-}$, and WT mice after the injection of LPS (30 mg/kg). Kaplan-Meier analysis demonstrated a significant difference in survival between $\text{I}\kappa\text{B}\beta^{-/-}$ and WT (log-rank test $\text{I}\kappa\text{B}\beta^{-/-}$ versus WT; $P < 0.0001$). Data are from three separate experiments and the number of mice in each group is indicated.

constructs into RAW264.7 macrophages (Fig. 5 C). The -518 bp IL-1 β 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-1B) 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-1 β promoter in RAW264.7 macrophages. However, we cannot completely exclude the contribution of the distal κB site.

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

Recruitment of $\text{I}\kappa\text{B}\beta$ to the IL-1 β promoter in complex with NF- κB p65-c-Rel

To test whether $\text{I}\kappa\text{B}\beta$ directly binds to the IL-1 β promoter, we performed chromatin immunoprecipitation (ChIP) assays.

ChIP analysis using RAW264.7 macrophages demonstrated recruitment of $\text{I}\kappa\text{B}\beta$ to the κB site of IL-1 β promoter upon stimulation with LPS (Fig. 6 A). In contrast, $\text{I}\kappa\text{B}\beta$ was not recruited to NF- κB -binding site in the MIP-2 gene promoter, suggesting specific regulation of the IL-1 β promoter by $\text{I}\kappa\text{B}\beta$ (Widmer et al., 1993). To characterize the activation complex in more detail, we investigated p65, p50, p52, and c-Rel binding. In addition to $\text{I}\kappa\text{B}\beta$, we found recruitment of the p65-c-Rel complex to the IL-1 β promoter after LPS stimulation in RAW264.7 macrophages, suggesting that a p65-c-Rel dimer, assembled by $\text{I}\kappa\text{B}\beta$, induces IL-1 β 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 $\text{I}\kappa\text{B}\beta$ was observed, demonstrating specificity for the IL-1 β promoter (Fig. 6 A). Functionality of c-Rel for LPS-induced IL-1 β transcription was also demonstrated using RNA interference in RAW264.7 macrophages (Fig. S3). To further demonstrate the influence of $\text{I}\kappa\text{B}\beta$ for dimer formation at the IL-1 β promoter at the genetic level, we used ChIP assays in $\text{I}\kappa\text{B}\beta^{-/-}$ BMDMs. Binding of $\text{I}\kappa\text{B}\beta$ to the IL-1 β promoter upon LPS treatment was confirmed in BMDMs (Fig. 6 B). Whereas, c-Rel binds to the IL-1 β promoter in WT macrophages, reduced binding was observed in $\text{I}\kappa\text{B}\beta^{-/-}$ BMDMs (Fig. 6 B). A significant lower binding of RNA polymerase II to the IL-1 β promoter indicated decreased transcriptional activity in $\text{I}\kappa\text{B}\beta^{-/-}$ 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 $\text{I}\kappa\text{B}\beta^{-/-}$ BMDMs, confirming specificity for the IL-1 β promoter (Fig. 6 B). Consistent with a nuclear function of $\text{I}\kappa\text{B}\beta$, we observed nuclear accumulation of $\text{I}\kappa\text{B}\beta$ in LPS-treated BMDMs (Fig. S4). Previous studies demonstrated the interaction of $\text{I}\kappa\text{B}\beta$ with c-Rel-p65 complexes in stimulated WEHI 231 cells in the nucleus (Phillips and Ghosh, 1997). To investigate direct interaction of the $\text{I}\kappa\text{B}\beta$ -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-1 β promoter. In nuclear extracts an $\text{I}\kappa\text{B}\beta$ -p65-c-Rel trimer was found bound to the proximal κB binding site of the IL-1 β promoter after LPS treatment (Fig. 6 C).

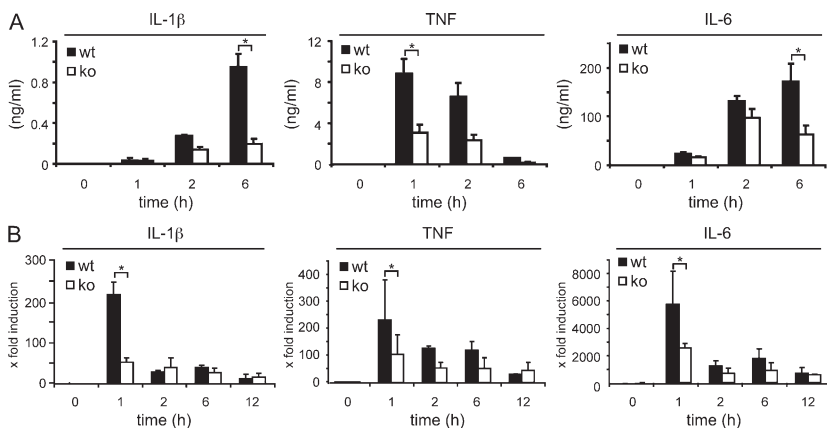


Figure 3. Deficiency in $\text{I}\kappa\text{B}\beta$ reduces LPS-triggered production of sepsis inducing cytokines.

(A) Serum levels of IL-1 β , TNF, and IL-6 in $\text{I}\kappa\text{B}\beta^{-/-}$ and control mice after LPS injection (30 mg/kg). TNF, IL-1 β , 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-1 β , 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 κ B oligonucleotide in the assay used. Altogether, these data suggest that I κ B β is recruited to the IL-1 β promoter after LPS treatment and is needed for NF- κ B complex formation and transcriptional activation.

DISCUSSION

Several reports of mice with targeted disruptions of I κ B family members demonstrated that the different proteins play distinct biological roles. In contrast to I κ B α , much less is known regarding to the *in vivo* function of I κ B β . In this study, we analyzed I κ B β ^{-/-} mice and demonstrate a novel function of I κ B β in the whole organism. I κ B β ^{-/-} mice share none of the hallmarks compared with I κ B α ^{-/-} mice (Beg et al., 1995; Klement et al., 1996). Similar to I κ B ϵ ^{-/-} mice, I κ B β ^{-/-} mice survive to adulthood and show no overt abnormalities (Mémet et al., 1999). However, our results demonstrate that I κ B β 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-1 β , 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 like IL-1 β , TNF, and IL-6 needs to be tightly regulated during an inflammatory response. We now demonstrate that I κ B β is a critical regulator of LPS-induced septic shock. I κ B β deficiency confers LPS resistance *in vivo*, which is caused by the impaired secretion of the proinflammatory cytokines IL-1 β , TNF, and IL-6. In BMDM, IL-1 β was determined as a specific molecular I κ B β target, whereas the activation of the TNF and IL-6 genes remained unaffected after LPS treatment in this particular cellular model. Because IL-1 β ^{-/-} mice are not protected from high-dose, LPS-induced septic shock, other I κ B β 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-1 β converting enzyme-deficient mice, known to have neither detectable serum levels of IL-1 β nor IL-1 α upon LPS challenge (Li et al., 1995). Interestingly, in microarray analysis of LPS-treated BMDMs, IL-1 α induction after LPS stimulation was impaired in I κ B β ^{-/-} BMDMs (Table S1). However, Glaccum et al. (1997) reported that IL-1R^{-/-} mice, which are refractory to both IL-1 α and IL-1 β signaling, are not resistant to LPS-induced septic shock, indicating that additional genes must contribute to the resistance in I κ B β ^{-/-} 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 by LPS in BMDMs in an I κ B β -dependent manner, points to the possibility that a complex I κ B β -controlled genetic network mediates the LPS resistance observed in I κ B β ^{-/-} mice.

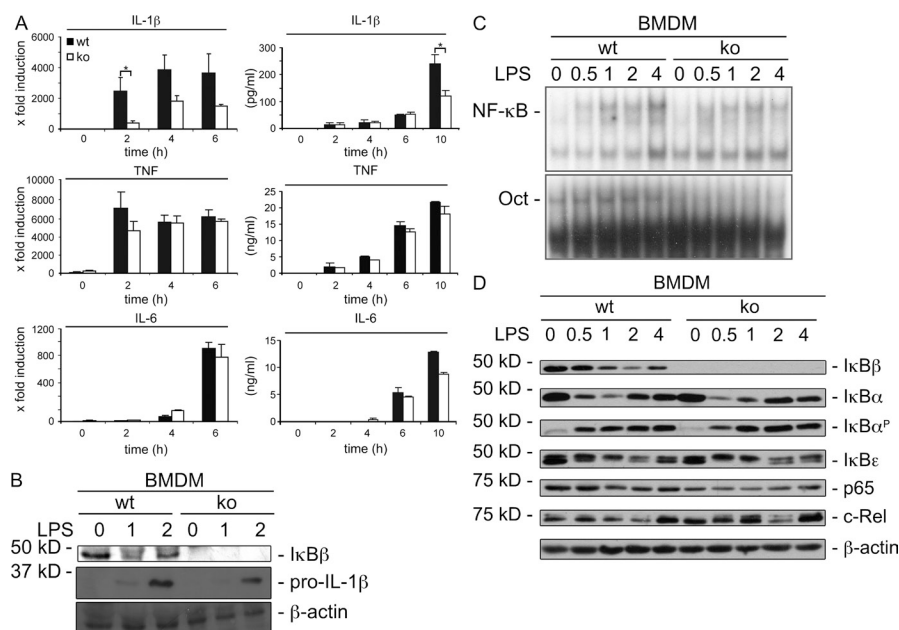


Figure 4. Knockout of I κ B β exhibit reduced IL-1 β expression in macrophages (BMDMs).

(A) WT and I κ B β ^{-/-} BMDM (three animals for each group) were stimulated with LPS (1 μ g/ml) as indicated. Total RNA was prepared and IL-1 β , TNF, and IL-6 mRNA levels were quantified using real-time PCR analysis (left; Student's *t* test; *, *P* < 0.01 versus control). IL-1 β , IL-6, and TNF cytokine secretion in response to LPS was determined by ELISA (right; Student's *t* test; *, *P* < 0.01 versus control). Results are shown as the mean of two independent experiments. (B) BMDMs from WT and I κ B β ^{-/-} mice were stimulated with LPS (1 μ g/ml) for indicated time points or were left as an untreated control. Western blot detects I κ B β and pro-IL-1 β expression. Similar results were obtained from two additional experiments. β -actin was used as loading control. (C) EMSA using a radiolabeled probe containing an NF- κ B-binding site. BMDMs were LPS-treated (100 ng/ml) as indicated and nuclear extracts were analyzed by EMSA. An Oct-

consensus oligonucleotide was used to control equal protein input. Three independent experiments revealed similar results. (D) BMDMs isolated from WT and I κ B β ^{-/-} mice were stimulated with LPS (100 ng/ml) and analyzed by Western blot. Expression levels of indicated NF- κ B proteins and I κ B members were determined at the indicated time points. One out of three independent experiments is shown.

Analysis of the $\text{I}\kappa\text{B}\beta$ -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 $\text{I}\kappa\text{B}\beta$ (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-1 β secretion was impaired because of $\text{I}\kappa\text{B}\beta$ deficiency, whereas the LPS-induced expression of IL-1 β , TNF, and IL-6 was dependent on $\text{I}\kappa\text{B}\beta$ 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-1 β , TNF, and IL-6 (Koo et al., 1999). Therefore, we cannot exclude that a different set of genes controlled by $\text{I}\kappa\text{B}\beta$ in response to LPS in Kupfer cells, including IL-1 β , TNF, and IL-6, are responsible for the LPS-resistance observed in $\text{I}\kappa\text{B}\beta^{-/-}$ 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 leukoprotease inhibitor-deficient mice are characterized by an increased NF- κB signaling magnitude, and the LPS-resistant poly ADP-ribose polymerase-1-deficient mice demonstrate a distinct impaired NF- κB activation (Oliver et al., 1999; Nakamura et al., 2003). $\text{I}\kappa\text{B}\beta$ is thought to control late-phase NF- κB activation (Hoffmann et al., 2002). This $\text{I}\kappa\text{B}\beta$ activity was not observed in LPS-stimulated BMDMs because LPS stimulation results in similar NF- κB activation kinetics in WT and $\text{I}\kappa\text{B}\beta^{-/-}$ cells. Because $\text{I}\kappa\text{B}\epsilon$ is present in $\text{I}\kappa\text{B}\beta^{-/-}$ cells and degraded with the same kinetics as in WT BMDMs, $\text{I}\kappa\text{B}\epsilon$ may compensate for the $\text{I}\kappa\text{B}\beta$ loss.

We detected a gene-specific activator function of $\text{I}\kappa\text{B}\beta$ during the early LPS-induced NF- κB response. As a model of $\text{I}\kappa\text{B}\beta$ -dependent transcriptional regulation, we focused onto the control of the IL-1 β promoter because induction of this gene by LPS mostly depends on $\text{I}\kappa\text{B}\beta$, revealing a ten fold decreased inducibility in WT compared with $\text{I}\kappa\text{B}\beta^{-/-}$ BMDMs (Table S1). We observed a direct recruitment of $\text{I}\kappa\text{B}\beta$ to the κB -binding site of the IL-1 β promoter in complex with p65/c-Rel. Interestingly, in BMDMs of $\text{I}\kappa\text{B}\beta^{-/-}$ mice the lack of $\text{I}\kappa\text{B}\beta$ binding to the IL-1 β promoter leads to the loss of c-Rel recruitment as well as reduced binding of RNA polymerase II, indicating reduced transcriptional

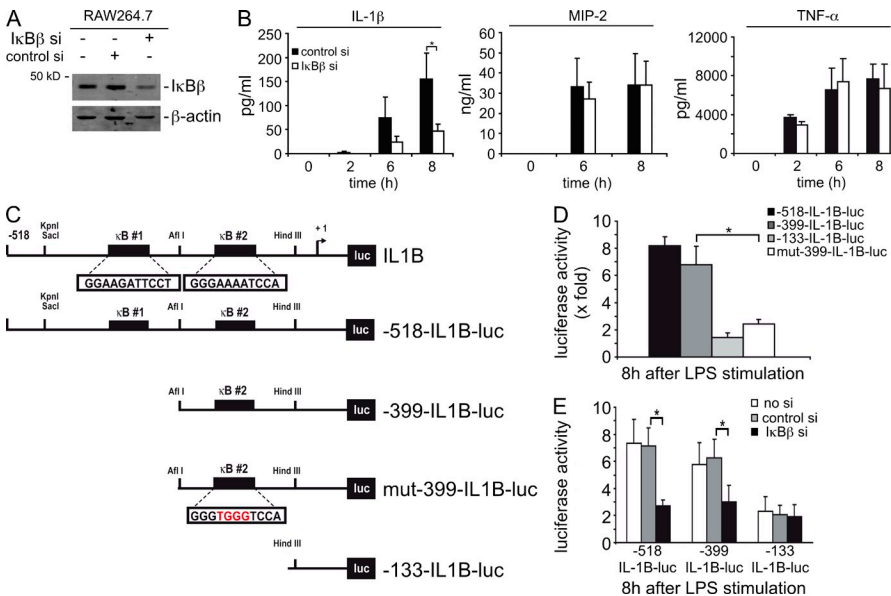


Figure 5. $\text{I}\kappa\text{B}\beta$ knock down results a significant reduction in IL-1 β cytokine expression and secretion. (A) Silencing of $\text{I}\kappa\text{B}\beta$ expression in RAW264.7 macrophages using siRNA. RAW264.7 macrophages were transfected with control or $\text{I}\kappa\text{B}\beta$ -specific siRNAs or were left as an untransfected control. After 48 h, Western blot detected $\text{I}\kappa\text{B}\beta$ expression. β -actin was used as loading control. (B) Measurement of IL-1 β , MIP-2, and TNF cytokine secretion in control and $\text{I}\kappa\text{B}\beta$ siRNA-transfected RAW264.7 cells. 48 h after transfection, cells were treated with LPS (100 ng/ml) and cytokine secretion was measured by ELISA. Data were obtained from three independent experiments performed in triplicate, and the results are presented as mean and SEM. (C) Schematic maps of different luciferase reporter genes containing IL-1 β promoter luciferase reporter gene constructs. Three different luc-constructs encoding IL-1 β promoter sequence starting from -518, -399,

and -133, respectively, were used in transient transfection assays in RAW264.7 macrophages. The IL-1 β -Luc construct IL-1B-518 includes two functional κB sites, whereas the IL-1B-399 contains only one functional κB site. No κB sites are present in the -133 IL-1 β -Luc construct. In the mut-399-IL1B-luc construct, a mutation was introduced into the κB site by site-directed mutagenesis, as indicated. (D) RAW264.7 macrophages were transfected with 1 μg of the indicated IL-1 β promoter luciferase reporter gene constructs. 48 h after the transfection, cells were stimulated with LPS (100 ng/ml) for 8 h and luciferase activity was determined. Data were obtained from three independent experiments performed in triplicate, and the results are presented as mean and SEM. (E) RAW264.7 macrophages were cotransfected using 1 μg of the indicated IL-1 β promoter luciferase reporter gene constructs, a control siRNA, an $\text{I}\kappa\text{B}\beta$ -specific siRNA, or they were left as an untransfected control. 48 h after the transfection, cells were stimulated with LPS (100 ng/ml) for 8 h and luciferase activity was determined. Data were obtained from three independent experiments performed in triplicate, and the results are presented as mean and SEM.

activation. Therefore, the remaining p65/RelA homodimers are not sufficient to activate IL-1 β transcription, suggesting that I κ B β is indispensable in formation of a transcriptional active p65-c-Rel complex at the IL-1 β promoter. This is in line with recent observations, demonstrating that each NF- κ B dimer supports a different amount of transcriptional activation at a specific gene promoter and that the IL-1 β gene-promoter is most responsive to p65/RelA and c-Rel in vitro (Algarte et al., 1999; Hiscott et al., 1993; Lin et al., 1995; Sacconi et al., 2003).

As previously characterized, a stable complex of I κ B β and NF- κ B p65/c-Rel can be found in the nucleus of WEHI 231 cells (Phillips and Ghosh, 1997). Using an oligonucleotide with specific κ B-binding sequence, we can also identify a NF- κ B p65-c-Rel-I κ B β complex in the nucleus of RAW264.7 macrophages after treatment with LPS, suggesting that I κ B β exists in the nucleus of macrophages, as found constitutively in WEHI 231 cells and LPS-stimulated 70/Z3 cells (Phillips and Ghosh, 1997; Suyang et al., 1996). Consistently, nuclear translocation of I κ B β was observed in LPS-stimulated BMDMs. Because I κ B β is clearly needed to recruit c-Rel to the IL-1 β promoter, we suggest a more active role for I κ B β in gene transcription than a sole chaperone function.

In addition to the inhibitory function of the I κ B protein family in resting cells, promoter-specific functions are becoming more evident. LPS signaling induces expression of I κ B ζ 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-I κ B ζ complex. Interestingly, LPS induction of IL-6 in macrophages was not shown to be I κ B β dependent (Yamamoto et al., 2004). Together with our data, which demonstrates

specificity of I κ B β toward certain NF- κ B- and MyD88-regulated promoters and recruitment of a p65/c-Rel dimer to the IL-1 β promoter, we propose that the I κ B proteins function to confer selectivity in NF- κ B dimer usage, and therefore in signaling specificity. In addition to I κ B β and I κ B ζ , I κ B α was shown to repress the *hes1* promoter by direct binding (Aguilera et al., 2004). Furthermore, it was demonstrated that I κ B α interacts with corepressors, like SMRT and N-CoR and different histone deacetylases (Aguilera et al., 2004). Whether I κ B β interacts with the epigenetic machinery is unknown in the moment and awaits further experiments.

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

MATERIALS AND METHODS

Targeted disruption of the I κ B β gene. The 8.2-kb genomic clone containing exons 3–6 of *I κ B β* was isolated from a genomic 129/Sv λ -DASHII-phage library. In the targeting vector, a loxP-flanked PGK-*neomycin* (*neo*)-cassette was introduced into intron 3. An additional loxP site was cloned into the AvrII site in intron 5. Thereby the BamHI sites in intron 3 and intron 5 were destroyed. The HSV-*thymidine kinase* gene was inserted 3.5 kb downstream of the *neo*-cassette. Embryonic stem cells (line E 14.1) were electroporated with the linearized vector (10 μ g) and selected with G418 (300 μ g/ml; Biochrom) and gancyclovir (2 μ M; Sigma-Aldrich). Resistant clones were screened for homologous recombination by PCR. Positive clones were verified by Southern blot analysis using an external 5' flanking probe (exon 2) and a *neo* probe. Correctly targeted ES cells were electroporated with pi-Cre-Plasmid (10 μ g), to remove the *neo*-cassette, and selected with neomycin. Neomycin-sensitive

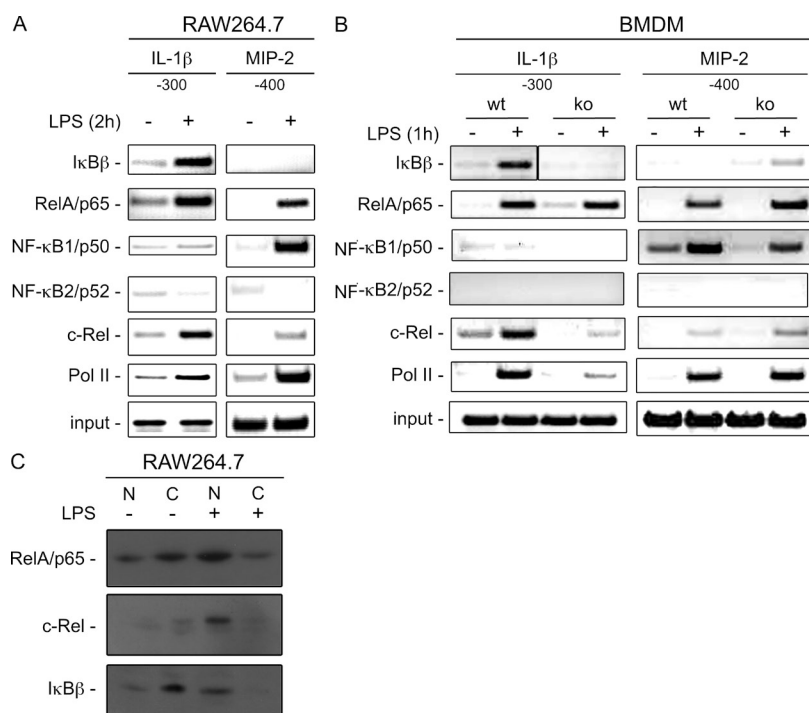


Figure 6. Recruitment of I κ B β to the IL-1 β promoter in complex with NF- κ B p65/c-Rel. (A) ChIP in RAW264.7 macrophages. RAW264.7 macrophages were stimulated for 2 h with LPS (100 ng/ml). Chromatin was immunoprecipitated with I κ B β -, RelA/p65-, NF- κ B1/p50-, NF- κ B2/p52-, c-Rel-, and RNA-polymerase 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-1 β 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 I κ B β -, RelA/p65-, NF- κ B1/p50-, NF- κ B2/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-1 β or MIP-2 promoters. Three independent experiments revealed similar results. (C) Biotin-streptavidin pull-down assay with a κ B oligonucleotide, corresponding to the proximal κ B site of the IL-1 β promoter. RAW264.7 macrophages were stimulated with LPS (100 ng/ml) for 2 h. Nuclear and cytoplasmic extracts were incubated with the κ B oligonucleotide and pulled down with streptavidin-agarose. Western blot detected RelA/p65, c-Rel, and I κ B β . 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 morulae, and resulting chimeric mice were crossed with *Deleter-Cre*-mice to generate *IκBβ* knockout mice (Schwenk et al., 1995). Disruption of the *IκBβ* gene was verified by Southern blot and PCR analyses of tail DNA. Homozygous offspring were obtained at the predicted frequency by interbreeding heterozygous mice. The null phenotype created by mutation of the *IκBβ* gene was confirmed by Western blot analysis of spleen extracts. For LPS injection, *IκBβ*^{-/-} 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 *IκBβ*^{-/-} mice. WT C57BL/6 (littermate controls or purchased from Charles River Laboratories), *IκBβ*^{+/-} littermates, and *IκBβ*^{-/-} 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 *IκBβ* and RelA/p65 in WT and *IκBβ*^{-/-} LPS or untreated BMDMs, cells were fixed in 4% formaldehyde (Sigma-Aldrich), permeabilized with 0.3% Triton-X (Sigma-Aldrich), and stained with *IκBβ*- and RelA/p65-specific primary antibodies (Santa Cruz Biotechnology, Inc.), followed by a FITC-labeled secondary antibody (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 system (AxioCam MRc; Carl Zeiss, Inc.). High-resolution images were captured and analyzed using AxioVision 4.3 software (Carl Zeiss, Inc.).

ChIP. ChIP assays were performed as previously described (Fritsche et al., 2009; Schneider et al., 2006, 2010). An equal amount of chromatin (50–100 μg) was used for each precipitation. The following antibodies were used: *IκBβ*, RelA/p65, c-Rel, p50, p52, RNA-Polymerase II, and control IgG, all from Santa Cruz Biotechnology. One twentieth of the precipitated chromatin was used for each PCR reaction. To ensure linearity, 28 to 38 cycles were performed, and one representative result is shown. Sequences of the promoter specific primers are as follows: IL-1β promoter (-347/-151): sense, 5'-TCCC-TGGAAATCAAGGGGTGG-3', antisense 5'-TCTGGGTGTGCATCT-ACGTGCC-3'; MIP-2 promoter (-433/-138): sense 5'-CAACAGTGTACT-TACGCAGACG-3', antisense 5'-CTAGCTGCCTGCCTCATTCTAC-3'.

Quantitative real-time RT-PCR. 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 using real-time PCR analysis (TaqMan, PE Applied Biosystems; Schneider et al., 2006; Fritsche et al., 2009). Sequences of the primers are: IL-1β, forward 5'-CTCAATG-GACAGAATATCAACCAACA-3' and reverse 5'-ACAGGACAG-GTATAGATTCTTTCCTTTG-3'; IL-6, forward 5'-TCGGAGGCT-TAATTACACATGTTCT-3' and reverse IL-6 R 5'-GCATCATCGTT-GTTCATACAATCA-3'; TNF, forward 5'-ATGAGAAGTTCCCAA-ATGGCC-3' and reverse 5'-TCCACTTGGTGGTTTCGCTACG-3'; Cyclophilin, forward 5'-ATGGTCAACCCACCGTGT-3' and reverse 5'-TCTGCTGTCTTTGGAACCTTGTG-3'.

Determination of cytokine secretion. Cytokine levels in blood sera and culture supernatants of IL-1β, TNF, and IL-6 were determined using commercially available ELISA kits, according to the manufacturer's instructions (R&D Systems).

Southern and Western blot analysis. 10 μg of genomic tail DNA was digested with BamH I, yielding 4.8 and 3.1 kb fragments for *IκBβ*^{-/-} and *IκBβ* WT alleles, respectively. DNA was separated on agarose gels and transferred to nitrocellulose membrane (GeneScreen Plus; DuPont). Hybridization was performed using hybridization buffer (1 M NaCl, 100 mg/ml dextran sulfate, 1% SDS, and 50 μg/ml salmon sperm DNA) and a Rediprime random primer labeling kit (GE Healthcare) with P³² α-dCTP-labeled probes.

Whole-cell lysates were prepared and Western blots and immunoprecipitations were done as previously described (Fritsche et al., 2009; Schneider et al., 2006, 2010). The following antibodies were used: *IκBβ*, p65, p50, p52, c-Rel, *IκBα*, phospho-*IκBα*, *IκBε* (Santa Cruz Biotechnology, Inc.), IL-1β (R&D Systems), and β-actin (Sigma-Aldrich). Proteins were detected by Odyssey Infrared Imaging System (Licor).

Cell isolation and culture. For generating BMDMs, mice were killed by cervical dislocation under ether anesthesia and BM was flushed from humerus, femur, and tibia of 6–8 mice. Cells were collected and washed as previously described (Ohashi et al., 2000). Pluznik medium containing RPMI 1640, 5% heat-inactivated horse serum (PAA), 15% FCS (PAA), 15% culture supernatant from M-CSF producing L929 cells (DSM ACC), and 1% P/S was used for cell culturing. After 6 d, the enriched macrophages were used for experiments as indicated.

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, biotinylated 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 × 10⁷ RAW264.7 macrophages were used for each time point. Nuclear and cytosolic 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 -261 to -270 of the IL-1β promoter, was used: 5'-ACCCCAGGAAAACCAATATT-3'.

IL-1β promoter reporter gene assay and mutagenesis. To determine IL-1β promoter activity the -518-IL1B-luc, -399-IL1B-luc, and -199-IL1B-luc luciferase reporter gene constructs were used. Point mutations within the -399-IL-1B-luc plasmid were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) in conjunction with the following oligonucleotides: 5'-CATTCTTCTAACGTGTGGAAATCCACTATTAT-GTGGAC-3' and 5'-GTCCACATTAACCTGGATTCCAACACGTTAG-AAGAATC-3'. Mutated residues are shown in Fig. 5 C. Mutations were confirmed by sequencing. 1 μg of each reporter gene constructs 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 *IκBβ* and c-Rel. RAW264.7 macrophages were transfected with siRNA duplex (Ambion) specific for mouse *IκBβ* or with scramble duplex in a concentration of 200 nM with Oligofectamine (Invitrogen) according to the manufacturer's instructions. The following siRNAs were used: *IκBβ*, 5'-GACUGGAGGCUACAACUAG-3'; c-Rel, 5'-AUAGCAUGUUGACAUCAGACAUAUCU-3'; control siRNA, 5'-CAGUCGCGUUUGCGACUGG-3'.

EMSA. EMSAs were performed as previously described (Arkan et al., 2001) using NF-κB (5'-AGTTGAGGGGACTTTCCAGGC-3' and 3'-TCAACTCCCCGAAAGGGTCCG-5') and Oct-1 (5'-TGTC-GAATGCAAATCACTAGAA-3' and 3'-ACAGCTTACGTTTGTAGTGA-TCTT-5') oligonucleotides.

Gene expression profiling. Gene expression profiling was performed as previously described (Reichert et al., 2007; Fritsche et al., 2009). Duplicates 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 reduced to <55% in $I\kappa B\beta^{-/-}$ 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 $I\kappa B\beta$ 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 IL-1 β secretion in response to NF- κ B activators in $I\kappa B\beta^{-/-}$ BMDMs. Fig. S3 shows that c-Rel knockdown results a significant reduction in IL-1 β cytokine expression and secretion. Fig. S4 shows that LPS induces the nuclear translocation of $I\kappa B\beta$ in BMDMs. Table S1 shows genes activated by LPS in BMDM in an $I\kappa B\beta$ -dependent way. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20100864/DC1>.

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