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IκBβ acts to both inhibit and activate gene expression at different stages of the inflammatory response

Ping Rao[1], **Mathew S. Hayden**[1,2], **Meixiao Long**[1,2], **Martin L. Scott**[3,4], **A. Philip West**[1], **Dekai Zhang**[1,5], **Andrea Oeckinghaus**[1,2], **Candace Lynch**[6], **Alexander Hoffmann**[6], **David Baltimore**[3], and **Sankar Ghosh**[1,2]

[1] Department of Immunobiology and Department of Molecular Biophysics & Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520, USA

[2] Department of Microbiology & Immunology, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA

[3] Department of Biology, California Institute of Technology, Pasadena, CA 91125, USA

[6] Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093, USA

Abstract

The activation of pro-inflammatory gene programs by nuclear factor-κB (NF-κB) is primarily regulated through cytoplasmic sequestration of NF-κB by the inhibitor of κB (IκB) family of proteins[1]. IκBβ, a major IκB isoform, can sequester NF-κB in the cytoplasm[2], although its biological role remains unclear. While cells lacking IκBβ have been reported[3],[4], *in vivo* studies have been limited and suggested redundancy between IκBα and IκBβ[5]. Like IκBα, IκBβ is also inducibly degraded, however upon stimulation by LPS, IκBβ is degraded slowly and resynthesized as a hypophosphorylated form that can be detected in the nucleus[6]–[11]. The crystal structure of IκBβ bound to p65 suggested this complex might bind DNA[12]. *In vitro*, hypophosphorylated IκBβ can bind DNA with p65 and cRel, and the DNA-bound NF-κB:IκBβ complexes are resistant to IκBα, suggesting hypophosphorylated, nuclear IκBβ may prolong the expression of certain genes[9]–[11]. We now report that *in vivo* IκBβ serves to both inhibit and facilitate the inflammatory response. IκBβ degradation releases NF-κB dimers which upregulate pro-inflammatory target genes such as tumor necrosis factor-α (TNFα). Surprisingly absence of IκBβ results in a dramatic reduction of TNFα in response to lipopolysaccharide (LPS) even though activation of NF-κB is normal. The inhibition of TNFα mRNA expression correlates with the absence of nuclear,

Correspondence and requests for materials should be addressed to: sg2715@columbia.edu.

[4]Current address: Merck Research Laboratories, Boston, MA 02115, USA

[5]Current address: Center for Extracellular Matrix Biology, Texas A & M University Institute of Biosciences and Technology, Houston, Texas 77030, USA

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Author contributions PR characterized the mice and performed the majority of the experiments, MSH performed the immunoprecipitation experiments and helped in writing the paper, ML performed CIA experiments, DZ and APW performed generation of BMDM cells, AO performed some experiments, MLS and DB generated the knockout mice, CL and AH performed the RNAse protection assays, and SG conceived of the study and wrote the paper.

hypophosphorylated-IκBβ bound to p65:c-Rel heterodimers at a specific κB site on the TNFα promoter. Therefore IκBβ acts through p65:c-Rel dimers to maintain prolonged expression of TNFα. As a result, IκBβ$^{-/-}$ mice are resistant to LPS-induced septic shock and collagen-induced arthritis. Blocking IκBβ might be a promising new strategy for selectively inhibiting the chronic phase of TNFα production during the inflammatory response.

To better understand the biological function of IκBβ we decided to study mice lacking the IκBβ gene. Homologous recombination was used to delete the majority of the IκBβ coding sequences (30–308 aa) including elements essential for binding to NF-κB (Supplementary Fig. 2)[6,12,13]. Absence of IκBβ was confirmed by immunoblotting of mouse embryonic fibroblasts (MEFs; Supplementary Fig. 2). Although IκBβ is expressed broadly including in hematopoietic organs (Supplementary Fig. 3a), the *IκBβ* knockout mice breed and develop normally without any obvious phenotypic defects.

NF-κB and IκB proteins function in an integrated network and hence reduced expression of one component may cause compensatory changes in levels of other proteins [14,15]. However, expression levels of IκBα, IκBε, p65, RelB, c-Rel, p105 and p100 were unaffected in *IκBβ*$^{-/-}$ mice (Supplementary Fig. 3b). Increased NF-κB activity has been observed in other IκB knockouts[16–18], and increased basal NF-κB reporter activity was observed in *IκBβ*$^{-/-}$ MEFs (Fig. 1a). Electrophoretic mobility shift assays (EMSA) demonstrated increased basal NF-κB activity in *IκBβ*$^{-/-}$ cells (60%) (Supplementary Fig. 3c). Conversely, overexpression of IκBβ inhibits NF-κB activation (Supplementary Fig. 3d). Thus IκBβ inhibits NF-κB and degradation or loss of IκBβ contributes to NF-κB activity. NF-κB reporter assays reveal that absolute NF-κB activity in response to LPS, IL-1β or TNFα is slightly higher in the *IκBβ*$^{-/-}$ than wild type (WT) cells (Fig. 1a). However, the kinetics of NF-κB activation by EMSA, and the pattern of IκB degradation by immunoblotting, in cells stimulated with LPS, IL-1β or TNFα were not demonstrably different in *IκBβ*$^{-/-}$ cells (Supplementary Fig. 4). Thus, loss of IκBβ results in a modest elevation in basal NF-κB activity, while inducible NF-κB activation is relatively unaffected.

NF-κB regulates the expression of many genes, in particular those involved in inflammation and immune responses[19]. To determine whether IκBβ has a role in the inflammatory response, *IκBβ*$^{-/-}$ and *IκBβ*$^{-/+}$ mice were challenged with LPS. Surprisingly, *IκBβ*$^{-/-}$ mice were significantly resistant to the induction of shock (Fig. 1b). We therefore examined the serum levels of the key acute phase cytokines TNFα, IL-1β and IL-6[20] following LPS injection. In wild type mice TNFα production peaked 1 hour after LPS injection, while IL-6 and IL-1β production peaked around 2 hours, in agreement with previous studies[21]. Although serum IL-6 and IL-1β were reduced (~25%) in the *IκBβ*$^{-/-}$ mice, the reduction of TNFα levels (>70%) was more striking (Fig. 1C). As the peak of serum TNFα precedes that of IL-1β and IL-6, it is likely that the reduction of IL-1β and IL-6 is secondary. As monocytes and macrophages are major sources for systemic TNFα, we analyzed LPS induced cytokines in thioglycollate-elicited peritoneal macrophages (TEPM). While equivalent macrophage populations were obtained from the mice (Supplementary Fig. 5a), TNFα, but not IL-6, production was drastically reduced in *IκBβ*$^{-/-}$ TEPM (Fig. 1d).

To understand how IκBβ affects TNFα synthesis we examined each step of TNFα production. Secreted TNFα was detectable by ELISA after 2 hours of LPS stimulation and by 4 hours was significantly impaired in *IκBβ*$^{-/-}$ TEPM (Fig. 2a). IL-6 production was equivalent (Fig. 2a). We examined the level of pro-TNFα by intracellular FACS and found there was very little pro-TNFα detected in the *IκBβ*$^{-/-}$ TEPMs even after 8 hours of LPS stimulation (Fig. 2b). The average amount of pro-TNFα produced was 2–3 fold higher in WT compared to *IκBβ*$^{-/-}$ TEPM (Fig. 2c). Consistent with this difference in protein levels, steady-state TNFα was decreased 2–6 fold in the *IκBβ*$^{-/-}$ TEPM compared to WT cells (Fig. 2d). Although TNFα mRNA is known to be regulated[22],[23], there was no difference in TNFα mRNA stability between WT and *IκBβ*$^{-/-}$ TEPM (Supplementary Fig. 5b). Therefore, IκBβ promotes TNFα transcription.

To understand how IκBβ affects TNFα transcription, we investigated which NF-κB subunits were associated with IκBβ in macrophages. It is known that IκBβ associates with p65:p50 and c-Rel:p50 complexes[24] through direct binding to p65 and c-Rel but not p50[6]. However, we found that IκBβ could be immunoprecipitated only with p65 and c-Rel, but not p50 (Fig. 3a). Both immunoprecipitations with anti-p65 and anti-c-Rel antibodies pull down IκBβ, IκBα and p50. Thus, there are p65:p50 and inducible c-Rel:p50 complexes that are associated with IκBα or other IκBs, but not IκBβ. Reciprocal immunoprecipitation of p65 with c-Rel and both p65 and c-Rel with IκBβ suggests a p65:c-Rel heterodimer associated with IκBβ (Fig. 3b). To demonstrate the association of IκBβ with p65:c-Rel, we performed sequential immunoprecipitations by first immunoprecipitating IκBβ and then immunprecipitating the eluted IκBβ complexes with anti-c-Rel antibody. The presence of p65 in the anti-c-Rel immunoprecipitate confirms the presence of IκBβ:p65:c-Rel complex (Fig. 3c). The IκBβ:p65:c-Rel complex was found in nuclear extracts suggesting that this could be a transcriptionally active complex. We had previously reported[10] that IκBβ exists in two phosphorylation states: a hyperphosphorylated state in quiescent, unstimulated cells, and a hypophosphorylated newly synthesized state in LPS stimulated cells (Fig. 3c and Supplementary Fig. 5a). In the co-immunoprecipitation experiments shown here we found that both forms of IκBβ can bind p65 and c-Rel, although the hypophosphorylated form predominates in the IκBβ:p65:cRel complex following LPS stimulation.

There are four κB sites upstream of TNFα coding region, three of which are crucial for NF-κB dependent TNFα expression[25]. Therefore, we performed chromatin immunoprecipitation (ChIP) with anti-p65, anti-c-Rel and anti-IκBβ antibodies in RAW264.7 cells and monitored the region encompassing these three κB sites. Following LPS stimulation, TNFα promoter region DNA is enriched by p65, c-Rel and IκBβ antibodies by 56, 70 and 7 fold respectively (Fig. 3d). In contrast, IκBβ is not recruited to the IL-6 promoter following LPS stimulation while p65 and c-Rel are recruited as expected (Fig 3d). Recruitment of p65, c-Rel and IκBβ to the TNFα promoter was also confirmed in WT bone marrow derived macrophages (BMDM; Fig 3e). In the *IκBβ*$^{-/-}$ BMDM, both p65 and c-Rel are recruited normally to the TNFα promoter. However, when we performed immunoprecipitation with anti-p65, c-Rel and IκBβ are pulled down in WT but not *IκBβ*$^{-/-}$ BMDM (Fig. 3f). Therefore, p65 and c-Rel fail to form a stable complex in *IκBβ*$^{-/-}$ cells. Thus, the p65 and c-Rel recruited to the TNFα promoter in *IκBβ*$^{-/-}$ cells is not a p65:c-Rel complex. These data suggest that optimal

TNFα transcription requires a ternary complex of IκBβ:p65:c-Rel binding to the TNFα promoter.

In order to identify the κB site for p65:c-Rel binding we performed EMSAs using the three κB sites from the TNFα promoter as probes (κB2, κB2a and κB3, Supplementary Fig. 5b). We identified two distinct gel-shift patterns. κB3 and κB2a show two major bands (only κB3 is shown in Fig. 3g) while κB2 shows three major inducible shift bands. The components of the bands were identified by super-shift assay (Fig. 3g, right panel). The top band in the κB2 gel-shift is mostly p65:c-Rel. Interestingly, the κB2 site possesses features predicted to favor p65:c-Rel binding (Supplementary Fig. 5c). Similar κB binding sites in the CD40 and CXCL1 promoters also demonstrated coordinate recruitment of IκBβ, p65, and c-Rel (Supplementary Fig. 5d). Furthermore, deletion of the κB2 site from a TNFα promoter reporter abrogated IκBβ-dependent reporter gene expression (Supplementary Fig. 6). In *IκBβ*$^{-/-}$ BMDM, the p65:c-Rel complex binding to theκB2 in EMSA assays is missing (Fig. 3h), in agreement with the immunoprecipitation result. Therefore optimal TNFα transcription requires a p65:c-Rel complex, stabilized by hypophosphorylated IκBβ, binding to the κB2 site in the TNFα promoter.

To identify other genes affected by IκBβ deficiency, we examined gene expression profiles in WT and *IκBβ*$^{-/-}$ BMDM. As expected, TNFα and IκBβ are among the genes whose expression is affected by IκBβ deficiency while IL-6 and IL-1β are not affected (Fig. 4a). Of the genes whose expression is reduced in the *IκBβ*$^{-/-}$ cells we identified 14 with expression patterns resembling TNFα (Fig. 4b). The expression of these genes was also reduced in p65, c-Rel or p65/c-Rel knock-out fetal liver macrophages suggesting that LPS-induced expression of these genes might depend on a mechanism similar to TNFα (data not shown). The expression of TNFα, IL-1α, IL-6 and IL-1β in response to LPS was further examined by RNase protection (Fig. 4c) and qRT-PCR assays (Supplementary Fig. 7) demonstrating that the reduction in persistent expression of TNFα in *IκBβ*$^{-/-}$ cells is unique. Reduced *IL12b* mRNA and protein secretion in the knockout TEPM was confirmed by qRT-PCR (Fig. 4d) and ELISA (Fig. 4e). Notably, transcription of *IL12b*, which has a κB site similar to κB2 of TNFα (Supplementary Fig. 5c), has previously been shown to require c-Rel and be partially dependent on p65[26]. Thus, only a select group of NF-κB dependent genes are diminished similarly to TNFα upon IκBβ deletion. As TNFα plays a key role in inflammation, we wanted to test whether *IκBβ*$^{-/-}$ deletion would affect the course of inflammatory diseases.

Rheumatoid arthritis (RA) is a common inflammatory disease with morbidity resulting from ongoing release of pro-inflammatory cytokines, including TNFα, and consequent destruction of joint tissue[27]. Previous studies have shown that NF-κB plays a key role in mouse models of arthritis and blocking NF-κB has a dramatic effect in preventing disease[28],[29]. RA can also be effectively treated by anti-TNFα therapies, although there are significant side-effects[30]. The ability to block only persistent TNFα expression would be therapeutic without blocking beneficial TNFα responses including the expression of innate immune response genes. We therefore tested whether the lack of IκBβ altered the course of collagen-induced arthritis (CIA), a well-characterized mouse model of RA.

To induce CIA we immunized DBA/1J mice with bovine type II collagen. *IκBβ*$^{-/-}$ mice displayed delayed onset, lower incidence and decreased severity of CIA (Fig. 4f and Supplementary Fig. 8). Inflammation in the WT mice extended from the paws and digits to the ankle joints and distally through the limb (data not shown). In contrast, *IκBβ*$^{-/-}$ mice showed minimal visual signs of paw and joint swelling (Supplementary Fig. 8). Serum TNFα was markedly decreased in *IκBβ*$^{-/-}$ mice while other pro-inflammatory cytokines were not significantly affected (Fig. 4g and Supplementary Fig. 9). Therefore the absence of IκBβ limits the progression and severity of arthritis by reducing the chronic production of TNFα.

The results presented above demonstrate a dual role for IκBβ: during the early stages of LPS stimulation, NF-κB complexes released by IκBβ degradation contribute to the initial expression of TNFα (Supplementary Fig. 1). Then, newly synthesized hypophosphorylated IκBβ facilitates the formation of IκBβ:p65:c-Rel complexes which selectively bind to the κB2 site in the TNFα promoter augmenting transcription. As shown in the gene chip and RNAse protection assays, this is a relatively selective function and *IκBβ*$^{-/-}$ mice are, therefore, otherwise normal. Hence targeting IκBβ might be a promising new strategy to treat chronic inflammatory diseases such as arthritis.

Methods summary

Mice

IκBβ deficient mice were generated by standard homologous recombination in the CJ7 ES cell line using a targeting construct that replaced exon 2 through exon 5 with a G418-resistance gene. Screened ES cell clones were injected into blastocysts derived from C57BL/6 mice gave rise to IκBβ$^{-/+}$/IκBβ$^{+/+}$ chimeras. Germline transmission of the disrupted allele was obtained and verified by Southern blotting and PCR, and mice were backcrossed at least 10 generations onto the B57BL/6 background. Mice were backcrossed at least 8 generations onto the DBA background for CIA experiments. Mice were maintained in pathogen-free animal facilities at Yale Medical School.

Cells

WT and *IκBβ* knockout MEFs were generated from E12.5 embryos following timed breeding of IκBβ$^{+/-}$ animals. TEMPs were obtained from 6- to 8-week-old littermate mice three days after intraperitoneal injection with thioglycollate. BMDM were harvested by standard protocols and differentiated with 30% L929 supernatant-conditioned media.

Biochemistry

Cell fractionation, western blotting, EMSA, and immunoprecipitations were performed as previously described unless otherwise indicated[6].

LPS-induced shock

LPS-induced shock was tested by intraperitoneal injection of 50 ug/g body weight LPS and monitoring for survival. In a separate identical experiment, the mice were bled at 1 hr and 2

hr after LPS treatment and the concentration of TNF-α, IL-6 and IL-1β in the serum was measured by ELISA.

Intracellular cytokine analysis

Pro-TNFα levels were analyzed in LPS stimulated TEMPs cells following LPS stimulation and brefeldin-A treatment. TNFα was detected following cell permeabilization using standard intracellular cytokine staining and flow cytometry.

qRT-PCR

RNA expression was quantified by quantitative two-step SYBR real-time RT-PCR, and relative mRNA levels were obtained by normalizing the readout for each specific gene by that of β-actin.

Microarray Analysis

Microarrays for gene expression analyses were performed on BMDMs stimulated with LPS and Affymetrix Mouse genome 430A 2.0 arrays as per the manufacturers protocol.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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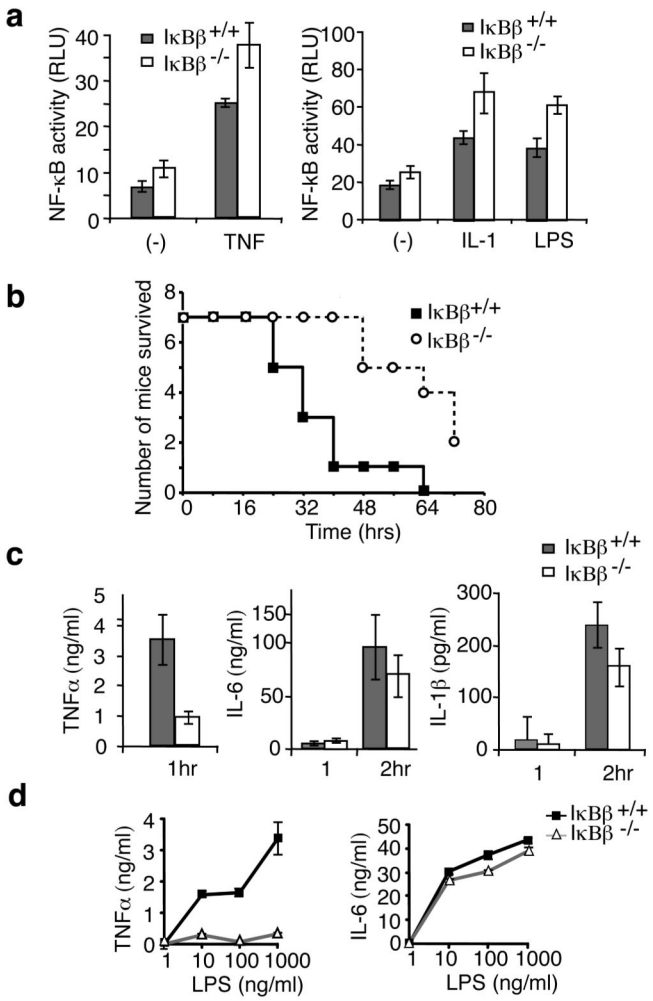

Figure 1. Mice lacking IκBβ are resistant to LPS-induced endotoxin shock

a, WT and *IκBβ*$^{-/-}$ MEF cells transfected with pBIIx-luc reporter and Renilla luciferase vectors were treated with TNFα, IL-1β or LPS for 4 hours and analyzed for luciferase activity. Results are expressed as relative luciferase unit (RLU) normalized by Renilla luciferase activity; error bars indicate ±s.d (n=3). **b,** Age and sex matched mice received intra-peritoneal injection of LPS and survival rates were scored every 8 hours for 3 days(n=7). **c**, Serum TNFα, IL-6 and IL-1β 1 hour and/or 2 hour after IP injection of LPS was examined by ELISA; error bars indicate ±s.d (n=5). **d**, TEPMs from littermate mice were treated for 20 hours with LPS as indicated, and TNFα and IL-6 in the media was determined by ELISA; error bars indicate ±s.d (n=3).

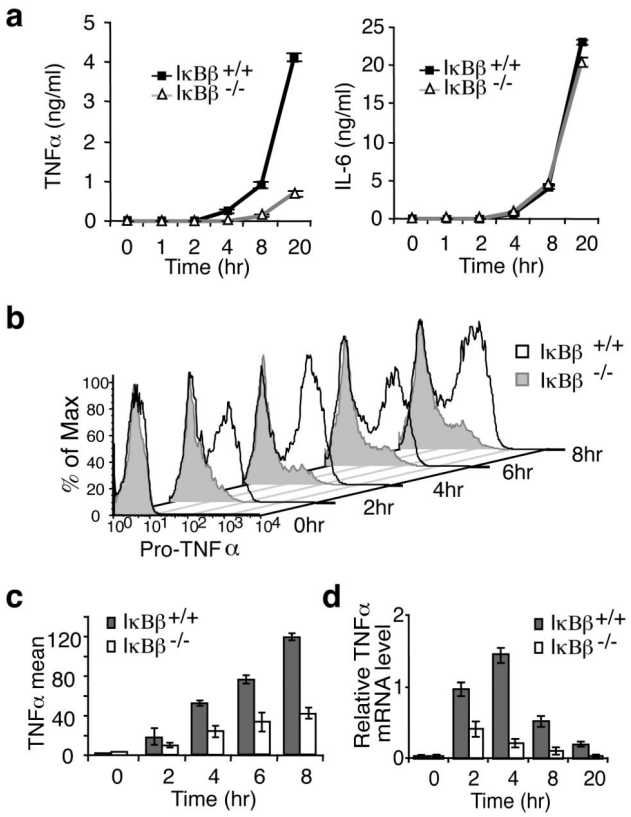

Figure 2. Deficient TNFα transcription in IκBβ$^{-/-}$ macrophages

a, TEPMs from littermate WT and *IκBβ*$^{-/-}$ mice were treated with LPS and secreted TNFα and IL-6 were determined by ELISA; error bars indicate ±s.d. (n=3). **b**, TEMPs from littermate mice were treated as in (a) in the presence of Brefeldin A, and intracellular pro-TNFα was examined with flow cytometry. **c**, Intracellular pro-TNFα production was examined as in B with macrophages isolated from 3 pairs of littermate mice; error bars indicate ±s.d. **d**, TEMPs were stimulated with LPS as in A and relative TNFα mRNA level was determined by qRT-PCR; error bars indicate ±s.d (n=3).

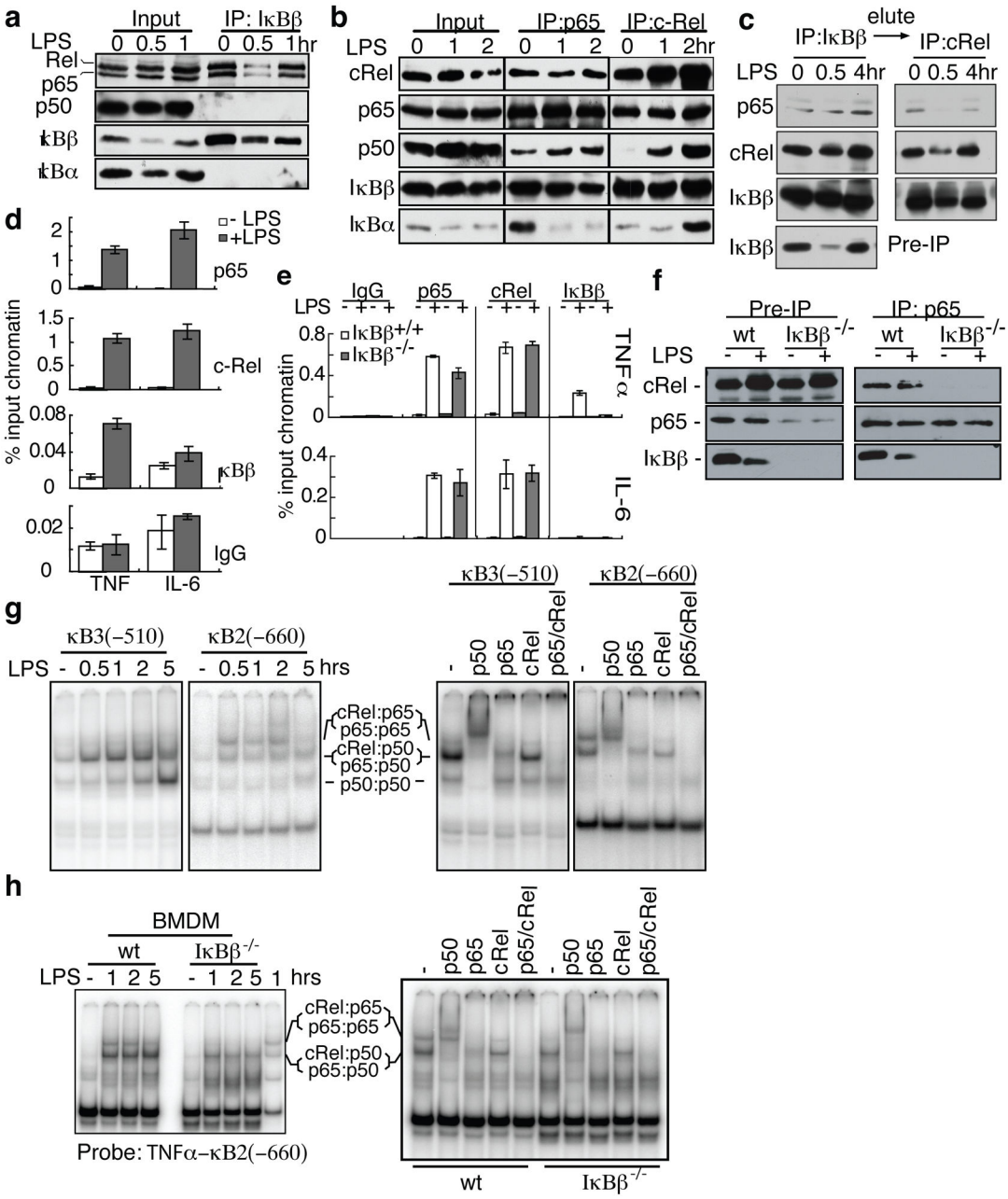

Figure 3. IκBβ is recruited to the promoter of TNFα together with P65 and c-Rel

a,b, Raw264.7 were stimulated with LPS and immunoprecipitated (IP) with anti-IκBβ (a), anti-p65 (b) or anti-c-Rel (b) antibodies and immunoblotted (IB) as indicated. **c,** LPS-stimulated Raw264.7 lysates were immunoprecipitated with anti-IκBβ; eluted with IκBβ peptide; immunoprecipitated with anti-c-Rel antibody; and immunoblotted as indicated. **d,** Raw264.7 lysates were subjected to ChIP as indicated and analyzed by qPCR targeting TNFα and IL-6 promoter κB sites; error bars indicate ±s.d (n=3). **e,** ChIP was performed as in (d) on WT and IκBβ$^{-/-}$ BMDM treated with LPS for 2 hours; error bars indicate ±s.d (n=3). **f**, BMDM treated as in (e) were immunoprecipitated with anti-p65 antibody. **g,** RAW264.7 were treated with LPS and nuclear extracts were subjected to EMSA TNFα κB3

or κB2 probes. Super shifts were performed using cells stimulated for 1hr. **h,** BMDM were treated with LPS and EMSA and supershifts with the κB2 probe were performed as in (g).

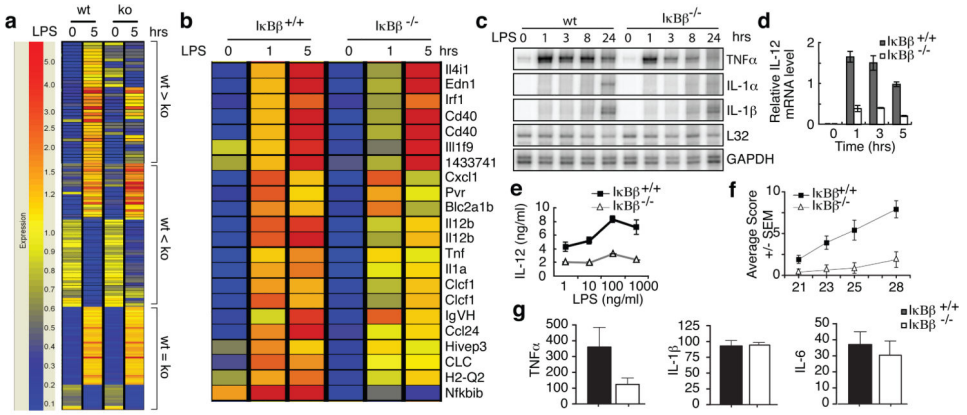

Figure 4. IκBβ knockout selectively affects only certain LPS responsive genes and attenuates collagen induced arthritis

a, LPS responsive genes whose expression is either down-regulated, up-regulated or unchanged in $I\kappa B\beta^{-/-}$ BMDM. **b,** Host-pathogen interaction genes that are IκBβ dependent and LPS responsive genes whose expression pattern resembles TNFα. **c,** RNase protection assay using WT and $I\kappa B\beta^{-/-}$ BMDM stimulated with LPS. **d,** IL-12b relative mRNA level determined by qRT-PCR in samples prepared as in (c); error bars indicate ±s.d. (n=3). **e,** ELISA for IL-12p40 secreted from WT and $I\kappa B\beta^{-/-}$ TEMP stimulated with LPS for 20 hours; error bars indicate ±s.d. **f,** Arthritis clinical scoring in WT (n=10) or $I\kappa B\beta^{-/-}$ (n=8) DBA mice; error bars indicate ±SEM. **g,** Serum TNF-α, IL-1β, and IL-6 levels in WT or $I\kappa B\beta^{-/-}$ DBA mice in (f); error bars indicate ±SEM.