LETTERS

IκBβ acts to inhibit and activate gene expression during the inflammatory response

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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 isoform of IκB, can sequester NF-κB in the cytoplasm[2], although its biological role remains unclear. Although 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 lipopolysaccharide (LPS), it is degraded slowly and re-synthesized 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 c-Rel, 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]. Here we report that *in vivo* IκBβ serves both to inhibit and facilitate the inflammatory response. IκBβ degradation releases NF-κB dimers which upregulate pro-inflammatory target genes such as tumour necrosis factor-α (TNF-α). Surprisingly, absence of IκBβ results in a dramatic reduction of TNF-α in response to LPS even though activation of NF-κB is normal. The inhibition of TNF-α messenger RNA (mRNA) expression correlates with the absence of nuclear, hypophosphorylated-IκBβ bound to p65:c-Rel heterodimers at a specific κB site on the TNF-α promoter. Therefore IκBβ acts through p65:c-Rel dimers to maintain prolonged expression of TNF-α. As a result, $I\kappa B\beta^{-/-}$ 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 TNF-α production during the inflammatory response.

To understand the biological function of IκBβ better, we studied mice lacking the *IκBβ* gene. Homologous recombination was used to delete most of the IκBβ coding sequences (30–308 amino acids) 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 haematopoietic 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. 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 $I\kappa B\beta^{-/-}$ 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 $I\kappa B\beta^{-/-}$ MEFs (Fig. 1a). Electrophoretic mobility shift assays (EMSAs) demonstrated increased basal NF-κB activity in $I\kappa B\beta^{-/-}$ 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 TNF-α is slightly higher in the $I\kappa B\beta^{-/-}$ than wild-type 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 TNF-α were not demonstrably different in $I\kappa B\beta^{-/-}$ cells (Supplementary Fig. 4). Thus, loss of IκBβ results in a modest elevation in basal NF-κB activity, whereas 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, $I\kappa B\beta^{-/-}$ and $I\kappa B\beta^{+/+}$ mice were challenged with LPS. Surprisingly, $I\kappa B\beta^{-/-}$ mice were significantly resistant to the induction of shock (Fig. 1b). We therefore examined the serum levels of the key acute phase cytokines TNF-α, IL-1β and IL-6 (ref. 20) after LPS injection. In wild-type mice TNF-α production peaked 1 h after LPS injection, whereas IL-6 and IL-1β production peaked around 2 h, in agreement with previous studies[21]. Although serum IL-6 and IL-1β were reduced (approximately 25%) in the $I\kappa B\beta^{-/-}$ mice, the reduction of TNF-α levels (greater than 70%) was more striking (Fig. 1c). As the peak of serum TNF-α 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 of systemic TNF-α, we analysed LPS-induced cytokines in thioglycollate-elicited peritoneal macrophages (TEPMs). Although equivalent macrophage populations were obtained from the mice (Supplementary Fig. 5a), production of TNF-α, but not IL-6, was drastically reduced in $I\kappa B\beta^{-/-}$ TEPMs (Fig. 1d).

To understand how IκBβ affects TNF-α synthesis we examined each step of TNF-α production. Secreted TNF-α was detectable by enzyme-linked immunosorbent assay (ELISA) after 2 h of LPS stimulation and by 4 h was significantly impaired in $I\kappa B\beta^{-/-}$ TEPMs (Fig. 2a). IL-6 production was equivalent (Fig. 2a). We examined the level of pro-TNF-α by intracellular fluorescence-activated cell sorting and found there was very little pro-TNF-α detected in the $I\kappa B\beta^{-/-}$ TEPMs, even after 8 h of LPS stimulation (Fig. 2b). The average amount of pro-TNF-α produced was two- to threefold higher in wild-type than $I\kappa B\beta^{-/-}$ TEPMs (Fig. 2c). Consistent with this

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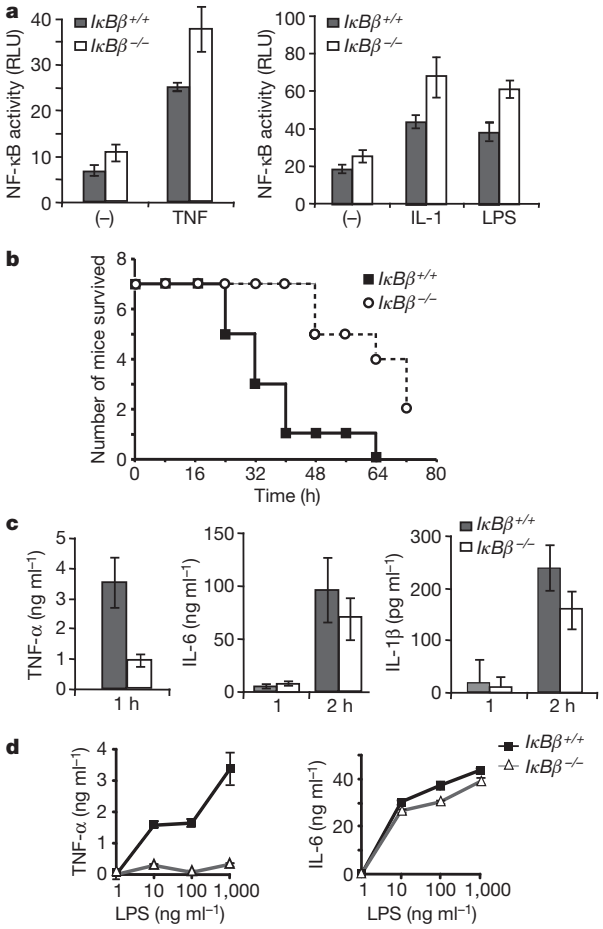

Figure 1 | Mice lacking IκBβ are resistant to LPS-induced endotoxin shock. **a**, Wild-type and $I\kappa B\beta^{-/-}$ MEF cells transfected with pBIIx-luc reporter and *Renilla* luciferase vectors were treated with TNF-α, IL-1β or LPS for 4 h and analysed for luciferase activity. Results are expressed as relative luciferase units (RLU) normalized by *Renilla* luciferase activity; error bars, s.d. ($n = 3$). **b**, Age- and sex-matched mice received intra-peritoneal injection of LPS and survival rates were scored every 8 h for 3 days ($n = 7$). **c**, Serum TNF-α, IL-6 and IL-1β 1 h and/or 2 h after intraperitoneal injection of LPS was examined by ELISA; error bars, s.d. ($n = 5$). **d**, TEPMs from littermate mice were treated for 20 h with LPS as indicated, and TNF-α and IL-6 in the media were determined by ELISA; error bars, s.d. ($n = 3$).

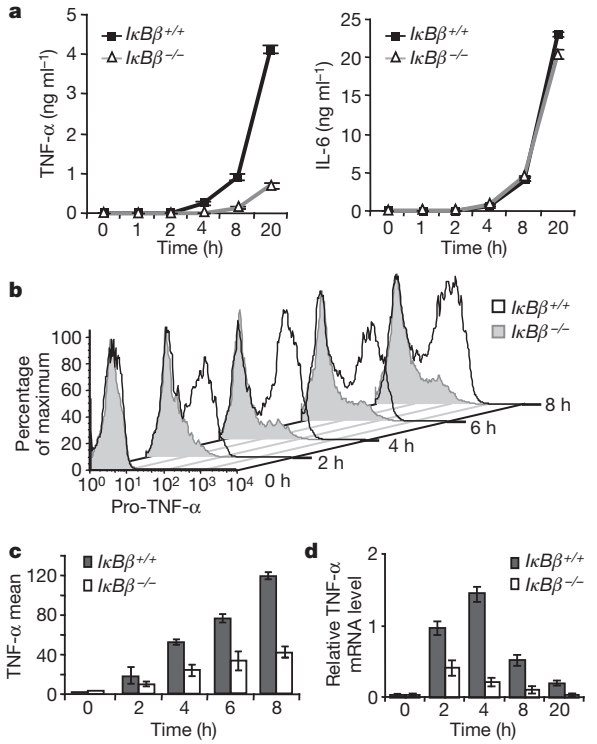

Figure 2 | Deficient TNF-α transcription in $I\kappa B\beta^{-/-}$ macrophages. **a**, TEPMs from littermate wild-type and $I\kappa B\beta^{-/-}$ mice were treated with LPS and secreted TNF-α and IL-6 were determined by ELISA; error bars, s.d. ($n = 3$). **b**, TEPMs from littermate mice were treated as in **a** in the presence of brefeldin A, and intracellular pro-TNF-α was examined with flow cytometry. **c**, Intracellular pro-TNF-α production was examined as in **b** with macrophages isolated from three pairs of littermate mice; error bars, s.d. **d**, TEPMs were stimulated with LPS as in **a** and relative TNF-α mRNA level was determined by qRT–PCR; error bars, s.d. ($n = 3$).

difference in protein levels, steady-state TNF-α mRNA was decreased two- to sixfold in the $I\kappa B\beta^{-/-}$ TEPMs compared with wild-type cells (Fig. 2d). Although TNF-α mRNA is known to be regulated post-transcriptionally[22,23], there was no difference in TNF-α mRNA stability between wild-type and $I\kappa B\beta^{-/-}$ TEPMs (Supplementary Fig. 5b). Therefore, IκBβ promotes TNF-α transcription.

To understand how IκBβ affects TNF-α 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 (ref. 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 immunoprecipitating the eluted IκBβ complexes with anti-c-Rel antibody. The presence of p65 in the anti-c-Rel immunoprecipitate confirms the presence of an IκBβ:p65:c-Rel complex (Fig. 3c).

The IκBβ:p65:c-Rel complex was found in nuclear extracts, which suggests 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 (Supplementary Fig. 6a). 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 after LPS stimulation.

There are four κB sites upstream of TNF-α coding region, three of which are crucial for NF-κB-dependent TNF-α expression[25]. Therefore, we performed chromatin immunoprecipitation with anti-p65, anti-c-Rel and anti-IκBβ antibodies in RAW264.7 cells and monitored the region encompassing these three κB sites. After LPS stimulation, TNF-α 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 after LPS stimulation whereas p65 and c-Rel are recruited as expected (Fig. 3d). Recruitment of p65, c-Rel and IκBβ to the TNF-α promoter was also confirmed in wild-type bone-marrow-derived macrophages (BMDMs; Fig. 3e). In the $I\kappa B\beta^{-/-}$ BMDM, both p65 and c-Rel are recruited normally to the TNF-α promoter. However, when we performed immunoprecipitation with anti-p65, c-Rel and IκBβ are pulled down in wild-type but not $I\kappa B\beta^{-/-}$ BMDMs (Fig. 3f). Therefore, p65 and c-Rel fail to form a stable complex in $I\kappa B\beta^{-/-}$ cells. Thus, the p65 and c-Rel recruited to the TNF-α promoter in $I\kappa B\beta^{-/-}$ cells are not a p65:c-Rel complex. These data suggest that optimal TNF-α transcription requires a ternary complex of IκBβ:p65:c-Rel binding to the TNF-α promoter.

To identify the κB site for p65:c-Rel binding we performed EMSAs using the three κB sites from the TNF-α promoter as probes (κB2, κB2a and κB3; Supplementary Fig. 6b). We identified two distinct

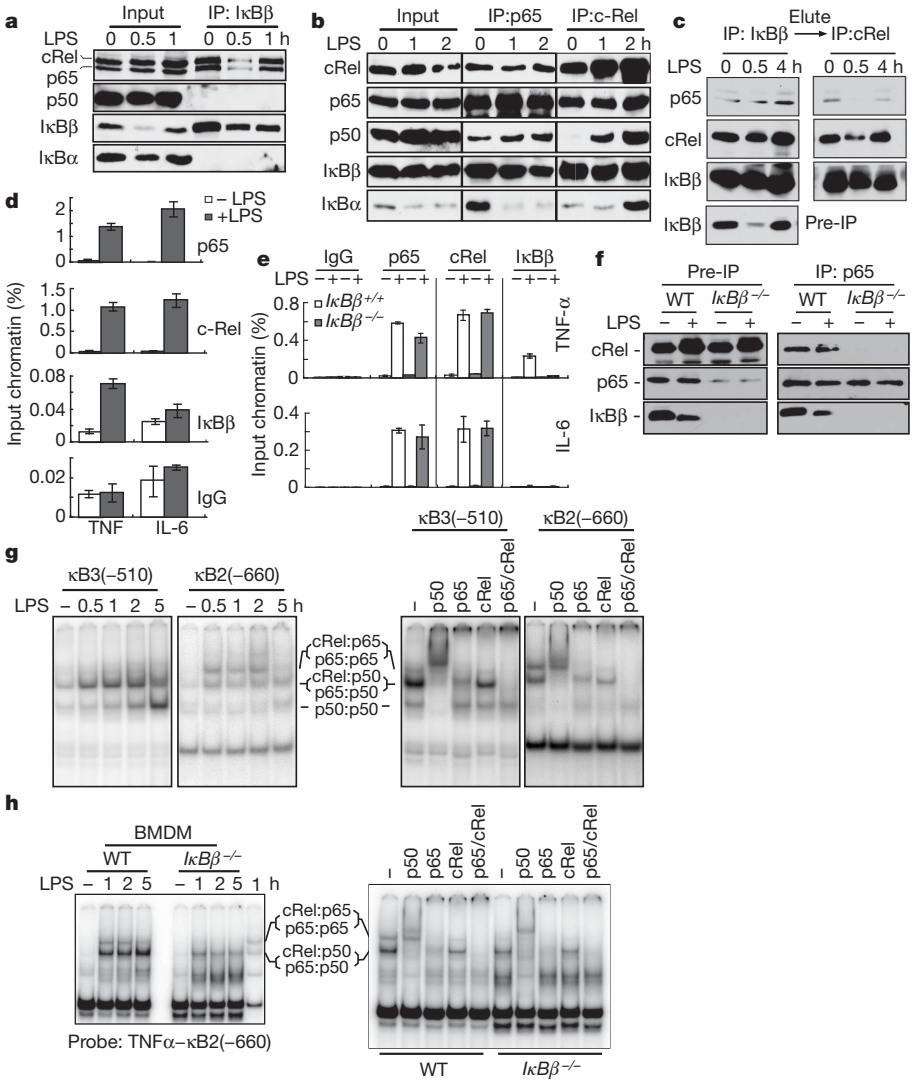

Figure 3 | IκBβ is recruited to the promoter of TNF-α with p65 and c-Rel. **a**, **b**, Raw264.7 were stimulated with LPS and immunoprecipitated (IP) with anti-IκBβ (**a**), anti-p65 (**b**) or anti-c-Rel (**b**) antibodies and immunoblotted as indicated. **c**, LPS-stimulated Raw264.7 lysates were immunoprecipitated with anti-IκBβ, eluted with IκBβ peptide, immunoprecipitated with anti-c-Rel antibody and immunoblotted as indicated. **d**, Raw264.7 lysates were subjected to chromatin immunoprecipitation as indicated and analysed by qPCR targeting TNF-α and IL-6 promoter κB sites; error bars, s.d. ($n = 3$). **e**, Chromatin immunoprecipitation was performed as in **d** on wild-type and $I\kappa B\beta^{-/-}$ BMDMs treated with LPS for 2 h; error bars, s.d. ($n = 3$). **f**, BMDMs treated as in **e** were immunoprecipitated with anti-p65 antibody. **g**, RAW264.7 were treated with LPS and nuclear extracts were subjected to EMSA with TNF-α κB3 or κB2 probes. Super shifts were performed using cells stimulated for 1 h. **h**, BMDMs were treated with LPS and EMSA and supershifts with the κB2 probe were performed as in **g**.

gel-shift patterns. κB3 and κB2a show two major bands (only κB3 is shown in Fig. 3g) whereas 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 favour p65:c-Rel binding (Supplementary Fig. 6c). Similar κB binding sites in the CD40 and CXCL1 promoters also demonstrated coordinate recruitment of IκBβ, p65 and c-Rel (Supplementary Fig. 6d). Furthermore, deletion of the κB2 site from a TNF-α promoter reporter abrogated IκBβ-dependent reporter gene expression (Supplementary Fig. 7). In $I\kappa B\beta^{-/-}$ BMDMs, the p65:c-Rel complex binding to the κB2 in EMSA assays is missing (Fig. 3h), in agreement with the immunoprecipitation result. Therefore optimal TNF-α transcription requires a p65:c-Rel complex, stabilized by hypophosphorylated IκBβ, binding to the κB2 site in the TNF-α promoter.

To identify other genes affected by IκBβ deficiency, we examined gene expression profiles in wild-type and $I\kappa B\beta^{-/-}$ BMDMs (Fig. 4a). As expected, *TNF-α* and *IκBβ* are among the genes whose expression is affected by IκBβ deficiency whereas IL-6 and IL-1β are not affected (Fig. 4b). Of the genes whose expression is reduced in the $I\kappa B\beta^{-/-}$ cells, we identified 14 with expression patterns resembling TNF-α (Fig. 4b). The expression of these genes was also reduced in p65, c-Rel or p65/c-Rel knockout fetal liver macrophages, which suggests that LPS-induced expression of these genes might depend on a mechanism similar to TNF-α (data not shown). The expression of TNF-α, IL-1α, IL-6 and IL-1β in response to LPS was further examined by RNase protection (Fig. 4c) and reverse transcription with quantitative real-time PCR (qRT–PCR) (Supplementary Fig. 8), which demonstrated that the reduction in persistent expression of TNF-α in $I\kappa B\beta^{-/-}$ cells is unique. Reduced *IL12b* mRNA and protein secretion in the knockout TEPMs was confirmed by qRT–PCR (Fig. 4d) and ELISA (Fig. 4e). Notably, transcription of *IL12b*, which has a κB site similar to κB2 of TNF-α (Supplementary Fig. 6c), has previously been shown to require c-Rel and be partly dependent on p65 (ref. 26). Thus, only a select group of NF-κB-dependent genes are diminished similarly to TNF-α upon IκBβ deletion. As TNF-α plays a key role in inflammation, we wanted to test whether $I\kappa B\beta^{-/-}$ deletion would affect the course of inflammatory diseases.

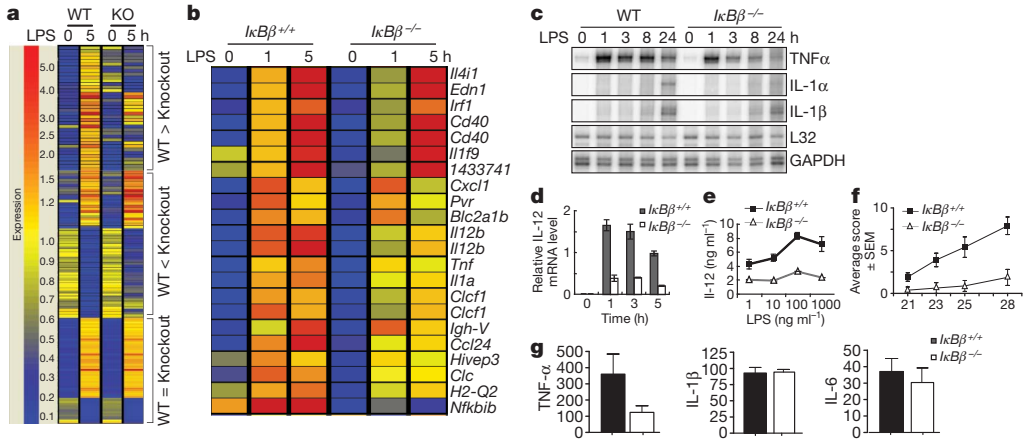

Figure 4 | IκBβ knockout selectively affects only certain LPS-responsive genes and attenuates collagen-induced arthritis. a, LPS-responsive genes whose expression is either downregulated, upregulated or unchanged in $I\kappa B\beta^{-/-}$ BMDMs. **b**, Host–pathogen interaction genes that are IκBβ dependent, LPS-responsive and whose expression pattern resembles TNF-α. **c**, RNase protection assay using wild-type and $I\kappa B\beta^{-/-}$ MEF stimulated with LPS. **d**, IL-12b relative mRNA level in TEMP determined by qRT–PCR; error bars, s.d. ($n = 3$). **e**, ELISA for IL-12p40 secreted from wild-type and $I\kappa B\beta^{-/-}$ TEPMs stimulated with LPS for 20 h; error bars, s.d. **f**, Arthritis clinical scoring in wild-type ($n = 10$) or $I\kappa B\beta^{-/-}$ ($n = 8$) DBA/1J mice; error bars, s.e.m. **g**, Serum TNF-α, IL-1β and IL-6 levels in wild-type or $I\kappa B\beta^{-/-}$ DBA/1J mice in **f**; error bars, s.e.m.

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

To induce collagen-induced arthritis, we immunized DBA/1J mice with bovine type II collagen. $I\kappa B\beta^{-/-}$ mice displayed delayed onset, lower incidence and decreased severity of collagen-induced arthritis (Fig. 4f and Supplementary Fig. 9). Inflammation in the wild-type mice extended from the paws and digits to the ankle joints and distally through the limb (data not shown). In contrast, $I\kappa B\beta^{-/-}$ mice showed minimal visual signs of paw and joint swelling (Supplementary Fig. 9c). Serum TNF-α was markedly decreased in $I\kappa B\beta^{-/-}$ mice whereas other pro-inflammatory cytokines were not significantly affected (Fig. 4g and Supplementary Fig. 10). Therefore the absence of IκBβ limits the progression and severity of arthritis by reducing the chronic production of TNF-α.

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 TNF-α (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 TNF-α promoter, augmenting transcription. As shown in the gene chip and RNase protection assays, this is a relatively selective function and $I\kappa B\beta^{-/-}$ 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 exons 2 to 5 with a G418-resistance gene. Screened ES cell clones were injected into blastocysts derived from C57BL/6 mice to give rise to $I\kappa B\beta^{-/+}/I\kappa B\beta^{+/+}$ chimaeras. Germline transmission of the disrupted allele was obtained and verified by Southern blotting and PCR, and mice were backcrossed at least ten generations onto the C57BL/6 background. Mice were backcrossed at least eight generations onto the DBA background for collagen-induced arthritis experiments. Mice were maintained in pathogen-free animal facilities at Yale Medical School.

Cells. Wild-type and *IκBβ* knockout MEFs were generated from embryos at embryonic day 12.5 after timed breeding of $I\kappa B\beta^{+/-}$ animals. TEPMs were obtained from 6- to 8-week-old littermate mice 3 days after intraperitoneal injection with thioglycollate. BMDMs were collected 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 μg g^{-1} body weight LPS and monitoring for survival. In a separate identical experiment, the mice were bled at 1 h and 2 h after LPS treatment and the concentrations of TNF-α, IL-6 and IL-1β in the serum were measured by ELISA.

Intracellular cytokine analysis. Pro-TNF-α levels were analysed in TEPMs after LPS stimulation and brefeldin-A treatment. TNF-α was detected after cell permeabilization by using standard intracellular cytokine staining and flow cytometry.

qRT–PCR. RNA expression was quantified by two-step SYBR qRT–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 manufacturer's protocol.

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

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions P.R. characterized the mice and performed most of the experiments, M.S.H. performed the immunoprecipitation experiments and helped in writing the paper, M.L. performed collagen-induced arthritis experiments, D.Z. and A.P.W. performed generation of BMDM cells, A.O. performed some experiments, M.L.S. and D.B. generated the knockout mice, C.L. and A.H. performed the RNAse protection assays, and S.G. conceived the study and wrote the paper.

Author Information The microarray data are deposited in National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE22223. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.G. (sg2715@columbia.edu).

METHODS

Mice. The IκBβ targeting construct contained the G418-resistance gene with recombination arm sequences derived from the genomic sequences flanking IκBβ exons 2 and 5 (Supplementary Fig. 2a). Homologous recombination between the targeting construct electroporated into the 129/SV mouse-derived ES cell line CJ7 and the endogenous IκBβ gene replaced the IκBβ sequences between exons 2 and 5 with the G418 resistance cassette. Homologous recombination was confirmed by hybridizing Southern blots of XbaI-digested ES DNA with probe, indicated in Supplementary Fig. 2a. Injection of mutant ES cell clones carrying the disrupted IκBβ gene into blastocysts derived from C57BL/6 mice gave rise to $I\kappa B\beta^{-/+}$/ $I\kappa B\beta^{+/+}$ chimaeras. Germline-transmittable $I\kappa B\beta^{-/+}$ mice were obtained by crossing chimaeras with C57BL/6 mice. $I\kappa B\beta^{-/+}$ mice (129SvEv background) were then backcrossed at least ten generations onto the C57BL/6 background before experiments. Mice used in the experiments were 6 to 8 weeks old derived by either brother–sister mating of $I\kappa B\beta^{-/-}$ or $I\kappa B\beta^{+/+}$ littermates (for age- and sex-matched mice experiments) or $I\kappa B\beta^{+/-}$ littermates (for littermate experiments). Backcrossed knockout and wild-type mice were maintained in pathogen-free animal facilities at Yale Medical School.

Generation of MEFs. Embryos at embryonic day 12.5 from timed breeding of $I\kappa B\beta^{-/+}$ female and male mice were dissected free of maternal tissues and Reichert's membrane, washed with PBS, sliced into small pieces and shaked with 0.05% trypsin-EDTA (GIBCO) for 30 min at 37 °C. The cells were suspended in DMEM supplemented with 10% fetal bovine serum and plated in 100 mm plates. Wild-type and $I\kappa B\beta^{-/-}$ MEFs were identified by immunoblotting with IκBβ antibody and PCR genotyping using MEF cells.

Antibodies and reagents. Antibodies used were anti-c-Rel, anti-RelB, anti-p52/p100, anti-p50/p105, anti- IκBα, anti-IκBβ, anti-IκBε (Santa Cruz), anti-GAPDH (Research Diagnostics), anti-p65 (BioMol), FITC-conjugated anti-TNF-α (eBiosciences), phycoerythrin-conjugated anti-CD11b and APC-conjugated anti-F4/80 (BD Biosciences). *Escherichia coli* LPS was purchased from Sigma-Aldrich (serotype 055:B5). In some experiments LPS from *Salmonella typhimurium* (Sigma-Aldrich) was used. Recombinant hTNF-α and mIL-1β used in MEF stimulation were from R & D Systems.

Cell fractionation. Cells were incubated in hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μg ml^{-1} leupeptin) at 4 °C for 15 min. Nonidet-40 was added to a final concentration of 0.05% and lysates were vortexed and centrifuged 1,000*g* for 5 min. Supernatants, containing the cytosolic fraction, were cleared by centrifugation at 20,000*g* for 10 min and were frozen at −80 °C. Nuclear pellets were washed with hypotonic lysis buffer and nuclear protein extracts for EMSA and nuclear immunoprecipitation were prepared by incubating the nuclei in hypertonic buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg ml^{-1} leupeptin) for 30 min with constant agitation at 4 °C. Nuclear lysates were then isolated after a 10 min spin at 13,000 rpm and frozen at −80 °C.

Western blot. Thirty to fifty micrograms of total protein were run on 12% SDS–polyacrylamide gel electrophoresis gel. After transfer to polyvinylidene fluoride membrane (Millipore), immunoblotting was performed as per the manufacturer's protocol.

EMSA assay. Five micrograms of protein from the nuclear fractions were incubated with ^{32}P-labelled double-stranded NF-κB or NF-Y probe (NF-κB: AGTTGAGGGGACTT TCCCAGG; NF-Y: ACTTTTAACCAATCAGAAAAAT) in binding buffer (5 mM Tris pH 7.5, 25 mM NaCl, 0.5 mM EDTA, 2.5% glycerol, 0.5 mM DTT, PolydI/dC 0.1 μg μl^{-1}, dGTP 3 mM, BSA 0.5 mg ml^{-1}) at room temperature for 15 min and run on a 6% non-denaturing polyacrylamide gel electrophoresis gel in 0.4 × TBE (36 mM Tris, 36 mM boric acid, 0.8 mM EDTA) buffer. Gel was visualized using Storage Phosphor Screen (Amersham Biosciences).

Luciferase assay. MEF cells (2.5×10^5) grown on 12-well plates were transiently transfected using FuGene 6 with the NF-κB-dependent reporter construct pBIIx-luc and the *Renilla* luciferase vector (Promega). Twenty-four hours after transfection, cells were stimulated with LPS (1 μg ml^{-1}), IL-1β (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) for 4 h. Unstimulated and stimulated cells were then lysed and luciferase activity was measured using the dual luciferase assay kit (Promega).

LPS-induced endotoxin shock. Age matched 8- to 12-week-old $I\kappa B\beta^{-/-}$ and control mice of both sexes were injected intraperitoneally with 50 μg g^{-1} body weight LPS (Sigma 55:011). Survival was examined every 8 h for up to 4 days. In a separate identical experiment, the mice were bled at 1 h and 2 h after LPS treatment and the concentration of TNF-α, IL-6 and IL-1β in the serum was measured by ELISA (BD Biosciences).

Thioglycollate-elicited peritoneal macrophages. Six- to 8-week-old littermate mice were injected intraperitoneally with 2 ml of 3% thioglycollate broth (Sigma). Three days later, the mice were killed and their peritoneal cavities were washed with 10 ml cold PBS (Gibco). Cell pellets were washed once with DMEM supplemented with 10% FBS and cultured at the concentration of 5×10^5 cells per millilitre. Two hours later the dishes were washed with medium to remove non-adherent cells. At least 75% of the remaining adherent cells were macrophages, as analysed by fluorescence-activated cell sorting (Supplementary Fig. 5a). Cells were cultured overnight in DMEM supplemented with only 0.5% FBS. Cells were then stimulated with LPS (1 μg ml^{-1}). TNF-α, IL-6 and IL-12 secreted in the medium were measured by ELISA. In some experiments cells were lysed and total RNA was prepared for qRT–PCR analysis of relative mRNA level.

Measurement of intracellular pro-TNF-α. Intracellular pro-TNF-α was measured in thioglycollate-elicited peritoneal macrophage stimulated with 1 μg ml^{-1} LPS for 2–8 h. To inhibit pro-TNF-α transportation to the plasma membrane and subsequent cleavage and release of secretive TNF-α, cells were treated with brefeldin A (eBioscience) while being stimulated by LPS. Cells were then scraped off the cell culture plates, washed in PBS and fixed with 1% formaldehyde. After being washed twice in PBS supplemented with 5% calf serum, cells were permeabilized by resuspending in BD Perm/Wash Buffer (BD Biosciences). Intracellular TNF-α was stained by incubating the permeabilized cells with FITC-anti-TNF-α antibody (eBiosciences) diluted in BD Perm/Wash Buffer for 30 min on ice. In some experiments, PE-anti-CD11b and APC-anti-F4/80 antibodies were incubated with TNF-α antibody. After washing with the Perm/Wash Buffer, stained cells were identified by flow cytometry.

BMDM cultures. Macrophages were derived from bone marrow following a standard protocol. Briefly, bone marrow cells were plated overnight to remove stromal cells and mature resident macrophages. Non-adherent cells were transferred to new plates and differentiated with 30% L929 supernatant-conditioned media over 7 days. Macrophages (7×10^5 cells per millilitre) were allowed to adhere overnight and stimulated with 1 μg ml^{-1} LPS.

qRT–PCR. RNAs were prepared using RNeasy Kit (Qiagene). RNA expression was quantified by two-step SYBR real-time RT–PCR (Stratagene). Relative mRNA level was obtained by normalizing the readout of a specific gene by that of β-actin. Oligonucleotide sequences used in quantitative PCR are available upon request.

Chromatin immunoprecipitation. RAW264.7 or BMDM cells were cross linked in 1% formaldehyde at room temperature for 7 min and lysed in RIPA buffer (10 mM Tris, pH 7.4, 0.3 M NaCl, 1 mM EDTA, 1% Triton-X 100, 0.1% NaDOC, 0.1% SDS, 1 mM PMSF, 1 μg ml^{-1} aprotinin and 1 μg ml^{-1} pepstatin A). After centrifugation at 1,700*g* for 5 min, the pellet nuclei were sonicated and chromatin immunoprecipitation was performed according to standard Upstate protocol. Immunoprecipitated chromatins were dissolved in 20 μl water and precipitated DNAs for the κB sites in TNF-α and IL-6 promoter were assayed by SYBR green qPCR with the following primers: TNF-α-fr: TGAGTTGATGTACCGC AGTCAAGA; TNF-α-bk: AGAGCAGCTTGAGAGTTGGGAAGT; IL6-fr: CGA TGCTAAACGACGTCACATTGTGCA; IL6-bk: CTCCAGAGCAGAATGAGCTAC AGACAT.

Microarray analysis. BMDMs were stimulated with LPS (1 μg ml^{-1}) and RNA was prepared with Qiagen RNeasy. Complementary DNA preparation and hybridization to Affymetrix Mouse Genome 430A 2.0 Arrays were performed at the Yale W.M. Keck facility.