

## RESEARCH ARTICLE

# Inhibiting I $\kappa$ B $\beta$ –NF $\kappa$ B signaling attenuates the expression of select pro-inflammatory genes

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**ABSTRACT**

Multiple mediators of septic shock are regulated by the transcription factor nuclear factor  $\kappa$ B (NF $\kappa$ B). However, complete NF $\kappa$ B inhibition can exacerbate disease, necessitating evaluation of targeted strategies to attenuate the pro-inflammatory response. Here, we demonstrate that in murine macrophages, low-dose NF $\kappa$ B inhibitors specifically attenuates lipopolysaccharide (LPS)-induced I $\kappa$ B $\beta$  degradation and the expression of a select subset of target genes (encoding IL1 $\beta$ , IL6, IL12 $\beta$ ). Gain- and loss-of-function experiments demonstrate the necessary and sufficient role of inhibitor of NF $\kappa$ B family member I $\kappa$ B $\beta$  (also known as NFKBIB) in the expression of these genes. Furthermore, both fibroblasts and macrophages isolated from I $\kappa$ B $\beta$  overexpressing mice demonstrate attenuated LPS-induced I $\kappa$ B $\beta$ –NF $\kappa$ B signaling and IL1 $\beta$ , IL6 and IL12 $\beta$  expression. Further confirming the role of I $\kappa$ B $\beta$  and its NF $\kappa$ B subunit binding partner cRel in LPS-induced gene expression, pre-treatment of wild-type mouse embryonic fibroblasts with a cell-permeable peptide containing the cRel nuclear localization sequence attenuated IL6 expression. We prove that LPS-induced I $\kappa$ B $\beta$ –NF $\kappa$ B signaling can be selectively modulated to attenuate the expression of select pro-inflammatory target genes, thus providing therapeutic insights for patients exposed to systemic inflammatory stress.

**KEY WORDS:** NF $\kappa$ B, I $\kappa$ B proteins, Lipopolysaccharide, LPS, Innate immune response

**INTRODUCTION**

Owing to its central role in regulating the expression of many pro-inflammatory genes, the transcription factor nuclear factor  $\kappa$ B (NF $\kappa$ B) has been implicated in the pathogenesis of septic shock (Liu and Malik, 2006). As such, NF $\kappa$ B inhibition has been proposed as a potential therapy for patients with septic shock (Rahman and Fazal, 2011; Wheeler et al., 2009). However, pre-clinical studies provide conflicting evidence on whether NF $\kappa$ B inhibition attenuates or exacerbates endotoxemic shock (Altavilla et al., 2002; Courtine et al., 2011; Everhart et al., 2006; Fujihara et al., 2000; Gadjeva et al., 2004; Greten et al., 2007; Kisseleva et al., 2006; Lawrence et al., 2005; Liu et al., 1997; Matsuda et al., 2004; Sha et al., 1995; Sheehan et al., 2002; Ulloa et al., 2002). It is not surprising that completely inhibiting NF $\kappa$ B activity would have both beneficial and detrimental consequences. However, it remains unclear whether the NF $\kappa$ B signaling cascade can be manipulated to attenuate the excessive and prolonged inflammation observed in patients with septic shock.

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Multiple factors dictate the NF $\kappa$ B transcriptional response to inflammatory stimuli. Understanding these factors might reveal therapeutic targets to attenuate – rather than completely inhibit – NF $\kappa$ B activity. The key step in canonical NF $\kappa$ B activation induced by inflammatory stimuli is phosphorylation and proteolysis of the inhibitor of NF $\kappa$ B (I $\kappa$ B) family of inhibitory proteins. In quiescent cells, members of the I $\kappa$ B family of NF $\kappa$ B inhibitory proteins, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ , keep inactivated NF $\kappa$ B dimers in the cytoplasm (Hayden and Ghosh, 2004). Following exposure to inflammatory stimuli [e.g. lipopolysaccharide (LPS)], the I $\kappa$ Bs are degraded, allowing NF $\kappa$ B nuclear translocation (Hayden and Ghosh, 2004). Although all cytoplasmic I $\kappa$ Bs inhibit NF $\kappa$ B activation, I $\kappa$ B $\beta$  plays a unique role in determining specific target gene expression. Because I $\kappa$ B $\beta$  preferentially binds cRel-containing NF $\kappa$ B dimers, and these dimer combinations bind to unique DNA sequences, specific downstream genes are targeted (Sen and Smale, 2010). Additionally, following degradation, both I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  are resynthesized and enter the nucleus. A nuclear export sequence (NES) found on I $\kappa$ B $\alpha$  allows it to export DNA-bound NF $\kappa$ B complexes from the nucleus (Hayden and Ghosh, 2004). In contrast, I $\kappa$ B $\beta$  lacks a NES, (Tam and Sen, 2001) and remains in the nucleus to stabilize NF $\kappa$ B–DNA binding (Sen and Smale, 2010). Thus, it is possible that targeting I $\kappa$ B $\beta$ -mediated NF $\kappa$ B activation could attenuate the persistent expression of specific target genes implicated in the pathogenesis of septic shock.

Studies in *Nfkbib*<sup>−/−</sup> (hereafter I $\kappa$ B $\beta$ <sup>−/−</sup>) mice support this hypothesis. When compared to wild-type (WT) mice exposed to a lethal dose of intraperitoneal LPS, I $\kappa$ B $\beta$ <sup>−/−</sup> mice show improved survival secondary to attenuated pro-inflammatory gene expression (encoding TNF $\alpha$ , IL1 $\beta$  and IL6) (Rao et al., 2010; Scheibel et al., 2010). Despite this, specific pharmacological inhibitors of I $\kappa$ B $\beta$ –NF $\kappa$ B have not been reported. Recently, we observed that pretreating fetal pulmonary endothelial cells with a low dose of parthenolide, a known NF $\kappa$ B inhibitor, attenuated LPS-induced I $\kappa$ B $\beta$  degradation, while not affecting I $\kappa$ B $\alpha$  degradation (Tang et al., 2013). Importantly, how specifically attenuating LPS-induced I $\kappa$ B $\beta$  degradation affects target gene expression is unknown.

Therefore, we hypothesized that inhibition of LPS-induced I $\kappa$ B $\beta$  degradation would result in attenuated expression of select NF $\kappa$ B target genes. We found that low-dose NF $\kappa$ B inhibitors (BAY 11-7085 and parthenolide) attenuated only LPS-induced I $\kappa$ B $\beta$  degradation in RAW 264.7 macrophages. Analysis of LPS-induced NF $\kappa$ B target gene expression revealed that a subset of genes (encoding IL1 $\beta$ , IL6 and IL12 $\beta$ ) were exquisitely sensitive to low-dose NF $\kappa$ B inhibitors. We next genetically modified I $\kappa$ B $\beta$  expression to confirm its mechanistic role in the expression of these specific NF $\kappa$ B target genes. Surprisingly, I $\kappa$ B $\beta$  overexpression in the absence of LPS activation was sufficient to significantly induce the expression of these same genes. The mechanism underlying this finding was that overexpression of I $\kappa$ B $\beta$  resulted in I $\kappa$ B $\beta$ –cRel nuclear translocation. Further implicating I $\kappa$ B $\beta$ –NF $\kappa$ B signaling in the expression of select

NF $\kappa$ B target genes, silencing I $\kappa$ B $\beta$  expression prior to LPS exposure significantly attenuated IL1 $\beta$ , IL6 and IL12 $\beta$  expression. Additionally, LPS-induced gene expression was assessed in mouse embryonic fibroblasts (MEFs) and bone-marrow-derived macrophages (BMDMs) isolated from I $\kappa$ B $\beta$  overexpressing (AKBI) mice. Consistent with cytoplasmic I $\kappa$ B $\beta$  overexpression, AKBI cells demonstrated abbreviated LPS-induced I $\kappa$ B $\beta$ –NF $\kappa$ B signaling and had attenuated IL1 $\beta$ , IL6 and IL12 $\beta$  expression. Further confirming a mechanistic role for I $\kappa$ B $\beta$  and its NF $\kappa$ B subunit binding partner cRel in LPS-induced gene expression, pre-treatment of WT MEFs with a cell-permeable peptide containing the cRel nuclear localization sequence attenuated IL6 expression. These results prove that pharmacologic inhibition of LPS-induced I $\kappa$ B $\beta$ –NF $\kappa$ B signaling attenuates expression of a specific subset of NF $\kappa$ B target genes. Further study will determine whether targeting this pathway is a viable option to improve the outcomes of patients exposed to systemic inflammatory stress.

## RESULTS

### The NF $\kappa$ B inhibitors BAY 11-7085 and parthenolide have dose-dependent effects on LPS-induced I $\kappa$ B degradation

We have previously reported that in fetal pulmonary artery endothelial cells low-dose parthenolide inhibits LPS-induced I $\kappa$ B $\beta$  degradation while not affecting I $\kappa$ B $\alpha$  degradation (Tang et al., 2013). To assess whether this was specific to fetal endothelial cells, RAW 264.7 murine macrophages were pretreated with low-dose BAY 11-7085 (1–5  $\mu$ M, 1 h) or parthenolide (1–5  $\mu$ M, 1 h) prior to LPS stimulation (30 min, 1  $\mu$ g/ml). At these doses, neither BAY 11-7085 nor parthenolide prevented LPS-induced I $\kappa$ B $\alpha$  degradation (Fig. 1A). In contrast, beginning at 1  $\mu$ M, both BAY 11-7085 and parthenolide attenuated I $\kappa$ B $\beta$  degradation (Fig. 1A). Consistent with previous reports, higher doses (10–20  $\mu$ M) of both BAY 11-7085 and parthenolide prevented I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  degradation (Fig. 1B). These data demonstrated that LPS-induced I $\kappa$ B $\beta$  degradation is more sensitive than I $\kappa$ B $\alpha$  to pharmacological inhibitory  $\kappa$ B kinase (IKK) inhibition. Selective inhibition of LPS-induced I $\kappa$ B $\beta$  degradation attenuated, but did not abolish, NF $\kappa$ B [the p65 subunit (also known as RelA) and cRel] nuclear translocation at later time points (Fig. 1C–E). These results show that a low dose of BAY 11-7085 and parthenolide prevent LPS-induced I $\kappa$ B $\beta$  degradation. Furthermore, specifically inhibiting I $\kappa$ B $\beta$ –NF $\kappa$ B signaling attenuates but does not completely inhibit nuclear translocation of NF $\kappa$ B subunits.

### Low-dose BAY 11-7085 and parthenolide attenuate LPS-induced expression of select NF $\kappa$ B target genes

Next, we evaluated the effect of low-dose NF $\kappa$ B inhibition on target gene expression. A selection of LPS-induced, MyD88-independent [encoding IP10 (also known as CXCR3), RANTES (also known as CCL5)] and MyD88-dependent (encoding IL1 $\beta$ , CXCL1, TNF $\alpha$ , IL12 $\beta$ , IL6) NF $\kappa$ B-dependent target genes were assessed (Björkbacka et al., 2004). Additionally, target genes that regulate NF $\kappa$ B activity [encoding I $\kappa$ B $\alpha$ , A20 (also known as TNFAIP3)] and antioxidant enzymes (MnSOD) were assessed (Morgan and Liu, 2011; Verstrepen et al., 2010). Three patterns of LPS-induced gene expression were noted with low-dose pharmacological NF $\kappa$ B inhibition. Low-dose parthenolide or BAY 11-7085 (1–5  $\mu$ M) did not affect LPS-induced expression of MnSOD and TNF $\alpha$  (Fig. 2A). In contrast, both BAY 11-7085 and parthenolide significantly attenuated LPS-induced expression of I $\kappa$ B $\alpha$ , A20, RANTES, CXCL1 and IP-10 beginning at 5  $\mu$ M (Fig. 2B). Finally, LPS-induced expression of IL1 $\beta$ , IL6 and IL12 $\beta$  was extremely sensitive

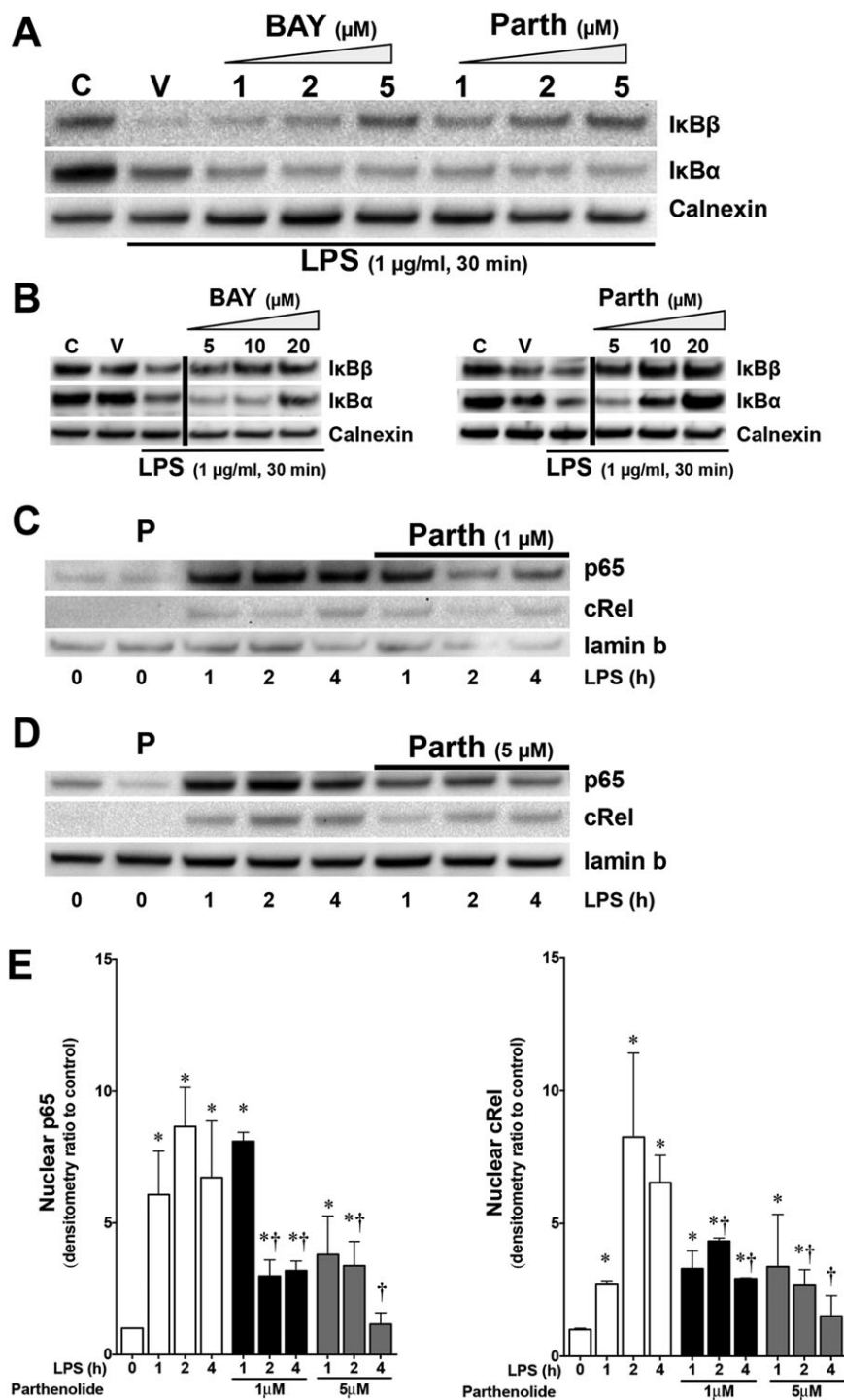
to the lowest dose of BAY 11-7085 and parthenolide tested (1  $\mu$ M, Fig. 2C). Of note, both parthenolide and BAY 11-7085 at this dose inhibited LPS-induced I $\kappa$ B $\beta$  degradation, without affecting I $\kappa$ B $\alpha$  degradation (Fig. 1A–C). Additionally, this dose of parthenolide only attenuates, but does not prevent NF $\kappa$ B nuclear translocation (Fig. 1D). These results suggest that I $\kappa$ B $\beta$  degradation is required for LPS-induced expression of a subset of NF $\kappa$ B target genes, and that this pathway can be pharmacologically manipulated to affect the expression of specific NF $\kappa$ B target genes.

### Nuclear I $\kappa$ B $\beta$ induces expression of target genes sensitive to low-dose pharmacologic NF $\kappa$ B blockade

It is possible that unrecognized, off-target effects of low-dose pharmacological NF $\kappa$ B inhibitors affected IL1 $\beta$ , IL6 and IL12 $\beta$  expression independently of I $\kappa$ B $\beta$  (Fig. 2C). Thus, we modified I $\kappa$ B $\beta$  expression in RAW 264.7 cells to define its mechanistic role in IL1 $\beta$ , IL6 and IL12 $\beta$  expression. Cells transfected with FLAG-tagged I $\kappa$ B $\beta$  expression plasmid demonstrated robust induction of I $\kappa$ B $\beta$  mRNA expression (Fig. 3A). Surprisingly, I $\kappa$ B $\beta$  overexpression – even in the absence of LPS activation – induced a robust transcriptional response of genes most sensitive to low-dose NF $\kappa$ B inhibition (IL6, IL1 $\beta$  and IL12 $\beta$ ) (Fig. 3B). This effect was not present with I $\kappa$ B $\alpha$  overexpression induced by transfection (supplementary material Fig. S1A). Of note, in the setting of transient I $\kappa$ B $\beta$  overexpression, LPS-induced expression of these genes was significantly attenuated, suggesting a common mechanism underlying LPS- and I $\kappa$ B $\beta$ -induced expression of these specific target genes (supplementary material Fig. S1B). In contrast to the effect observed on IL6, IL1 $\beta$  and IL12 $\beta$ , I $\kappa$ B $\beta$  overexpression had a variable effect on the expression of other target genes assessed (MnSOD, TNF $\alpha$ , I $\kappa$ B $\alpha$ , A20, RANTES, CXCL1, IP-10; Fig. 3B). Although cytoplasmic I $\kappa$ B $\beta$  is classically considered an inhibitory protein, nuclear I $\kappa$ B $\beta$  enhances gene expression (Rao et al., 2010). With this in mind, we sought to define the mechanisms underlying I $\kappa$ B $\beta$ -induced expression of select NF $\kappa$ B target genes. Western blot analysis confirmed that transfection with the I $\kappa$ B $\beta$  expression plasmid resulted in increased I $\kappa$ B $\beta$  protein expression (Fig. 3C, lane 2). Additionally, transfection alone was associated with nuclear translocation of I $\kappa$ B $\beta$ , cRel and p65 (Fig. 3C, lane 4). Of note, I $\kappa$ B $\beta$  overexpression modestly induced cRel, but not p65, expression (supplementary material Fig. S2). To determine whether cRel played a role in I $\kappa$ B $\beta$ -induced gene expression, RAW 264.7 cells were co-transfected with I $\kappa$ B $\beta$  expression plasmid and cRel small interfering RNA (siRNA) to achieve >50% reduction in cRel expression (Fig. 3D). Furthermore, macrophages co-transfected with the I $\kappa$ B $\beta$  expression plasmid and cRel siRNA demonstrated a ~50% decrease in IL1 $\beta$ , IL6 and IL12 $\beta$  expression when compared to I $\kappa$ B $\beta$ -transfection alone (Fig. 3E; unadjusted data available in supplementary material Fig. S3). Interestingly, similar levels of p65 knock-down did not significantly affect I $\kappa$ B $\beta$ -induced expression of these genes (Fig. 3F,G). These data demonstrate that I $\kappa$ B $\beta$  overexpression induces IL1 $\beta$ , IL6, IL12 $\beta$  expression through a cRel dependent mechanism.

### Silencing I $\kappa$ B $\beta$ expression attenuates LPS-induced expression of target genes sensitive to low-dose pharmacologic NF $\kappa$ B blockade

Having demonstrated that I $\kappa$ B $\beta$  overexpression induces IL1 $\beta$ , IL6 and IL12 $\beta$ , we sought to assess the effect of silencing I $\kappa$ B $\beta$  expression on LPS-induced gene expression. We reasoned that following cytosolic I $\kappa$ B $\beta$  degradation, re-accumulation and nuclear translocation of I $\kappa$ B $\beta$  would play mechanistic role in the LPS-induced expression of IL6, IL1 $\beta$  and IL12 $\beta$ . Thus, silencing I $\kappa$ B $\beta$



**Fig. 1. Pharmacological NF $\kappa$ B inhibitors have dose-dependent effects on LPS-induced I $\kappa$ B degradation in RAW 264.7 macrophages.** (A) Representative western blot showing I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in cytosolic fractions following exposure to LPS (30 min), or LPS after pretreatment (1 h) with BAY 11-7085 (1–5  $\mu\text{M}$ ) or parthenolide (1–5  $\mu\text{M}$ ) with calnexin as the loading control. (B) Representative western blot showing I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in cytosolic fractions following exposure to LPS (30 min), or LPS after pretreatment (1 h) with BAY 11-7085 (5–20  $\mu\text{M}$ ) or parthenolide (5–20  $\mu\text{M}$ ) with calnexin as the loading control. (C) Representative western blot showing cRel and p65 in nuclear extracts following exposure to LPS (1–4 h), or LPS after pretreatment (1 h) with parthenolide (1  $\mu\text{M}$ ) with lamin B as the loading control. (D) Representative western blot showing cRel and p65 in nuclear extracts following exposure to LPS (1–4 h), or LPS after pretreatment (1 h) with parthenolide (5  $\mu\text{M}$ ) with lamin B as the loading control. (E) Densitometric evaluation (mean $\pm$ s.e.m.) of nuclear p65 and cRel following exposure to LPS (1–4 h), or LPS after pretreatment (1 h) with parthenolide (1 or 5  $\mu\text{M}$ ).  $n=3$  per time point. \* $P<0.05$  versus unexposed control; † $P<0.05$  versus LPS-exposed cells. C, unexposed control; V, vehicle; P, parthenolide alone.

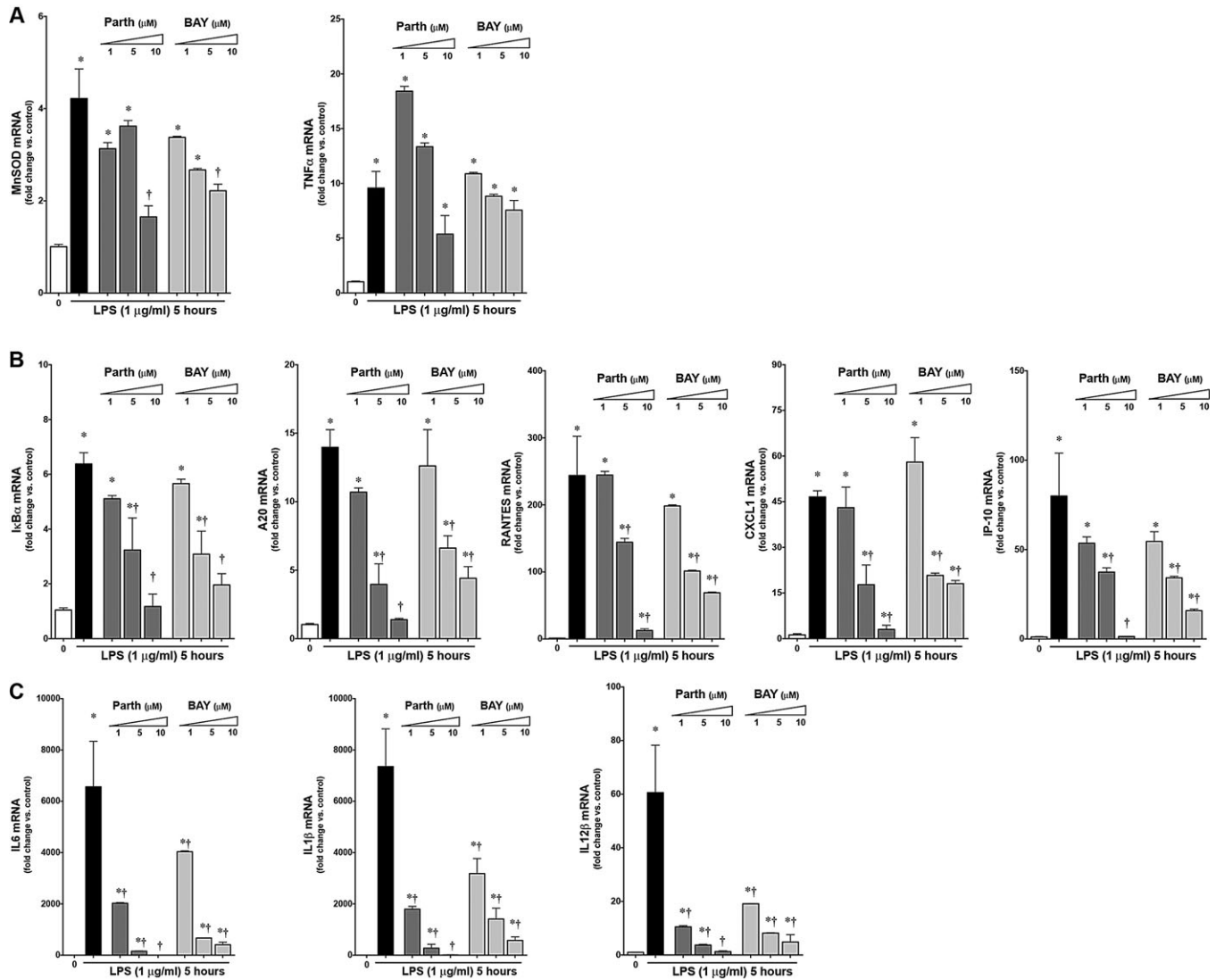
expression would attenuate expression of these genes. Following transfection of RAW 264.7 cells with I $\kappa$ B $\beta$  siRNA, western blotting and quantitative real-time PCR (qRT-PCR) confirmed a  $\sim 50\%$  reduction in I $\kappa$ B $\beta$  mRNA and protein expression (Fig. 4A,B). No significant effect on I $\kappa$ B $\alpha$  mRNA or protein expression was observed (Fig. 4A,B). Silencing I $\kappa$ B $\beta$  expression significantly reduced LPS-induced IL6, IL1 $\beta$  and IL12 $\beta$  expression (Fig. 4C; unadjusted data available in supplementary material Fig. S3). In contrast, silencing I $\kappa$ B $\beta$  had a variable effect on LPS-induced expression of other NF $\kappa$ B targets assessed (TNF $\alpha$ , MnSOD, I $\kappa$ B $\alpha$ , A20, RANTES, CXCL1 and IP-10; Fig. 4C; unadjusted data

available in supplementary material Fig. S3). These results show that there are specific NF $\kappa$ B target genes whose LPS-induced expression requires first cytosolic I $\kappa$ B $\beta$  degradation, followed by subsequent nuclear I $\kappa$ B $\beta$  translocation.

#### I $\kappa$ B $\beta$ knock-in mouse embryonic fibroblasts have shortened LPS-induced NF $\kappa$ B activation

To further establish a relationship between I $\kappa$ B $\beta$  and the expression of specific LPS-induced NF $\kappa$ B-regulated target genes, we performed experiments using MEFs derived from I $\kappa$ B $\beta$  knock-in (AKBI) mice. In AKBI mice, I $\kappa$ B $\beta$  cDNA replaces the I $\kappa$ B $\alpha$  gene,





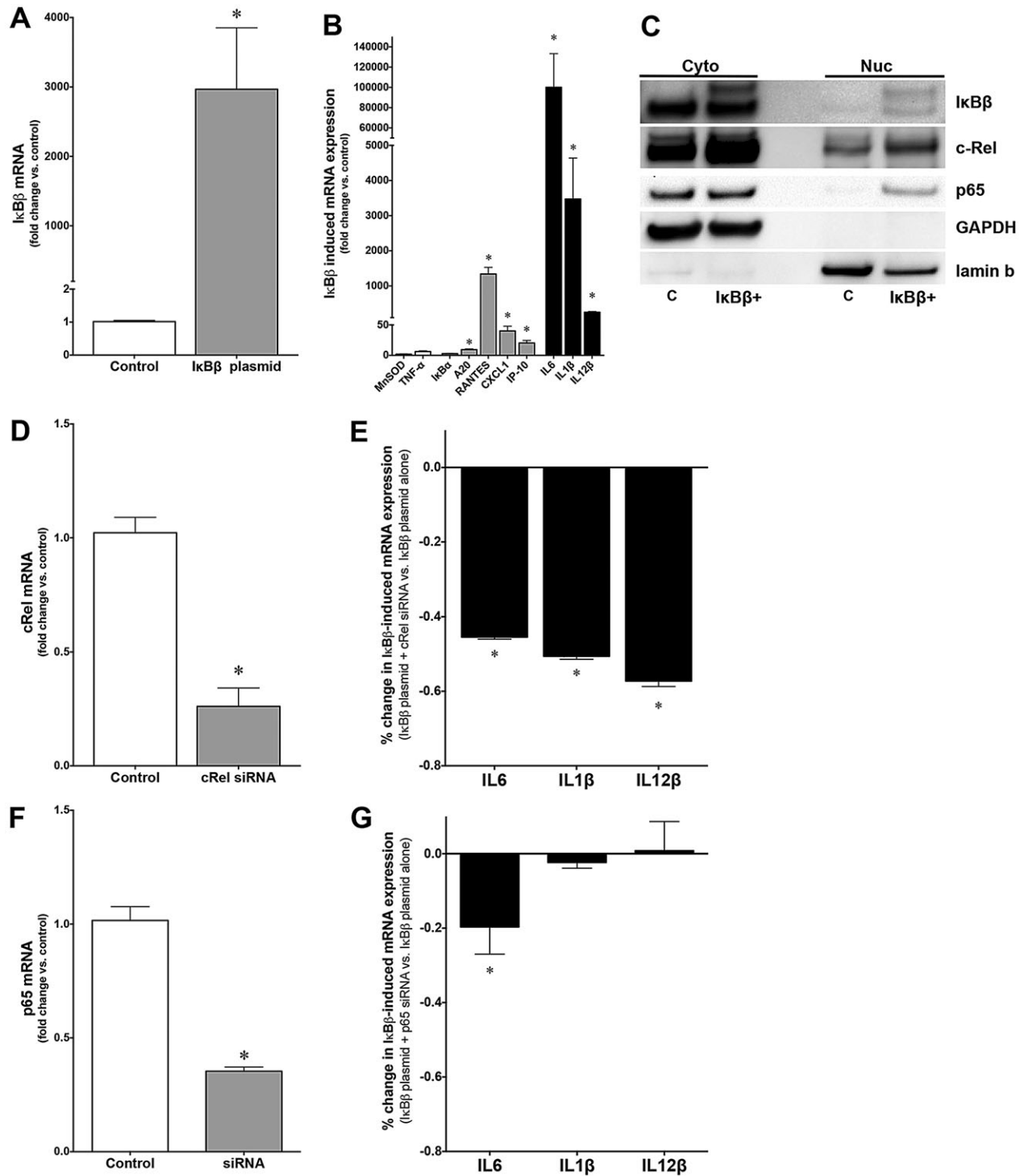
**Fig. 2. Low-dose pharmacological NF $\kappa$ B inhibitors have differing effects on LPS-induced NF $\kappa$ B target genes in RAW 264.7 macrophages.** Fold-increase of gene expression of (A) MnSOD and TNF $\alpha$ , (B) I $\kappa$ B $\alpha$ , A20, RANTES, CXCL1 and IP-10, and (C) IL1 $\beta$ , IL6 and IL12 $\beta$  following exposure to LPS (1  $\mu$ g/ml, 5 h), or LPS after pretreatment (1 h) with parthenolide (1, 5 or 10  $\mu$ M) or BAY 11-7085 (1, 5 or 10  $\mu$ M). Values are mean $\pm$ s.e.m.,  $n=3$  per time point. \* $P<0.05$  versus unexposed control; † $P<0.05$  versus LPS-exposed cells.

and thus these mice overexpress I $\kappa$ B $\beta$  and do not express I $\kappa$ B $\alpha$  (Cheng et al., 1998). Studies of these I $\kappa$ B $\beta$  knock-in, or AKBI, mice, showed that these mice were phenotypically indistinct from their wild-type controls. Furthermore, because the kinetics of TNF $\alpha$ -stimulated NF $\kappa$ B activity are similar between WT and AKBI MEFs (Cheng et al., 1998), it has been determined that I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  show 'functional redundancy'. Importantly, neither LPS-induced NF $\kappa$ B activity nor gene expression was assessed in that study (Cheng et al., 1998). First, because altered expression of the I $\kappa$ B family of proteins can affect expression of other NF $\kappa$ B family member proteins, we assessed the expression of key NF $\kappa$ B signaling proteins. In AKBI MEFs, expression of IKK $\alpha$ , IKK $\beta$ , IKK $\epsilon$ , p105 (large NF $\kappa$ B subunit encoded by *NFKB1*), p65, p50 (small NF $\kappa$ B subunit encoded by *NFKB1*), cRel, TLR4 and MyD88 was not different from WT cells by western blotting (supplementary material Fig. S4A) or qRT-PCR (supplementary material Fig. S4B). As expected, AKBI MEFs did not express I $\kappa$ B $\alpha$  and overexpressed I $\kappa$ B $\beta$  compared to WT MEFs (supplementary material Fig. S4A,B). These results show that the

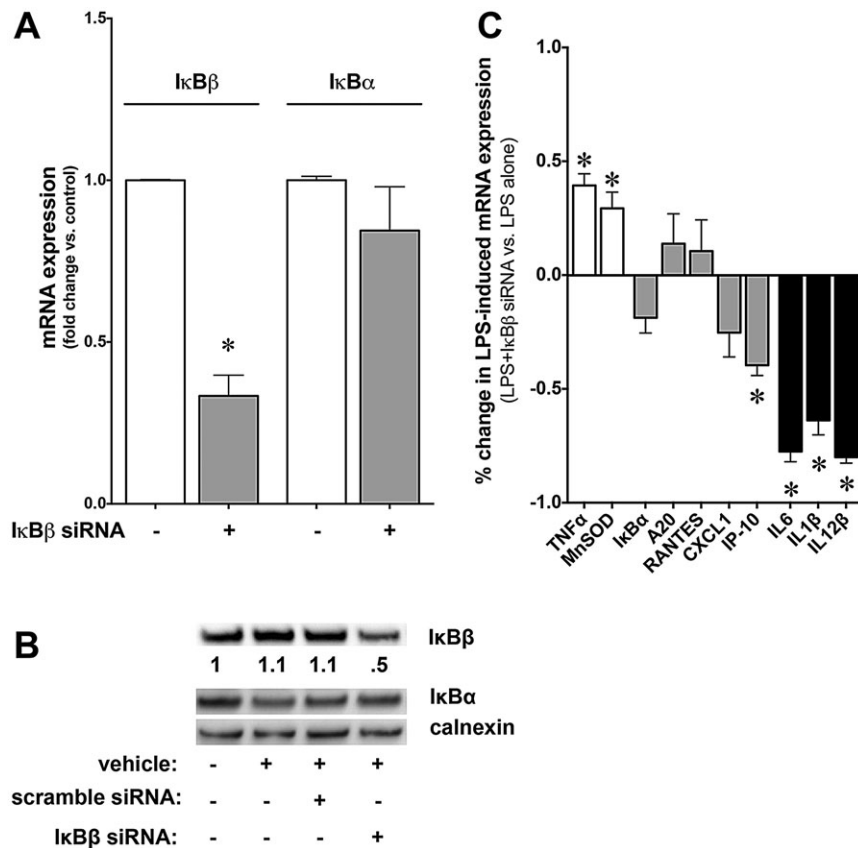
expression of key NF $\kappa$ B signaling proteins is similar between WT and AKBI MEFs.

The kinetics of LPS-induced NF $\kappa$ B activity was assessed in WT and AKBI MEFs. As expected, LPS-induced both I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  degradation in WT MEFs (Fig. 5A). Of note, in WT MEFs, I $\kappa$ B $\alpha$  re-accumulated by 2 h of exposure, whereas I $\kappa$ B $\beta$  levels remained significantly decreased throughout the 4-h exposure (Fig. 5A). Similarly, in AKBI MEFs, LPS induced I $\kappa$ B $\beta$  degradation by 1 h of exposure (Fig. 5A). However, in AKBI MEFs, cytosolic I $\kappa$ B $\beta$  re-accumulated within 2 h of exposure (Fig. 5A). This rapid re-accumulation is most likely due to the I $\kappa$ B $\beta$  knock-in construct, as I $\kappa$ B $\beta$  expression is driven by activity at the I $\kappa$ B $\alpha$  promoter (Cheng et al., 1998). These results prove abbreviated I $\kappa$ B $\beta$ -NF $\kappa$ B signaling in AKBI MEFs.

To assess the implications of rapid cytosolic re-accumulation of I $\kappa$ B $\beta$  following exposure to LPS, nuclear extracts were assessed for translocation of the NF $\kappa$ B subunits cRel, p65 and p50. In WT MEFs, LPS induced significant nuclear translocation of cRel, p65 and p50 after 1 h of exposure (Fig. 5B,C).



**Fig. 3. I $\kappa$ B $\beta$  overexpression causes cRel dependent expression of NF $\kappa$ B target genes.** (A) Relative I $\kappa$ B $\beta$  mRNA expression in RAW 264.7 macrophages following transfection with FLAG-tagged I $\kappa$ B $\beta$  expression plasmid. Values are means $\pm$ s.e.m.,  $n=9$ . \* $P$ <0.05 versus untransfected control. (B) Relative mRNA expression of target genes in RAW 264.7 macrophages following transfection with FLAG-tagged I $\kappa$ B $\beta$  expression plasmid. Values are means $\pm$ s.e.m.,  $n=6$ . \* $P$ <0.05 versus untransfected control. (C) Representative western blot showing I $\kappa$ B $\beta$ , cRel and p65 in cytosolic and nuclear extracts from RAW 267.4 macrophages transfected with I $\kappa$ B $\beta$  expression plasmid, with GAPDH as the cytosolic loading control and lamin B as the nuclear loading control. (D) Relative cRel mRNA expression in RAW 264.7 macrophages following transfection with cRel siRNA. Values are mean $\pm$ s.e.m.,  $n=6$ . \* $P$ <0.05 versus untransfected control. (E) Percentage change in I $\kappa$ B $\beta$ -induced gene expression in RAW 264.7 macrophages following co-transfection with cRel siRNA. Values are expressed as a ratio of the mRNA fold-change in cells transfected with the I $\kappa$ B $\beta$  expression plasmid with cRel siRNA transfected versus cells transfected with the I $\kappa$ B $\beta$  expression plasmid alone. Values are means $\pm$ s.e.m.,  $n=3$  per time point. \* $P$ <0.05 versus I $\kappa$ B $\beta$  expression plasmid alone. (F) Relative p65 mRNA expression in RAW 264.7 macrophages following transfection with p65 siRNA. Values are means $\pm$ s.e.m.,  $n=6$ . \* $P$ <0.05 versus untransfected control. (G) Percentage change in I $\kappa$ B $\beta$ -induced gene expression in RAW 264.7 macrophages following co-transfection with p65 siRNA. Values are expressed as a ratio of the mRNA fold-change in cells transfected with the I $\kappa$ B $\beta$  expression plasmid with cRel siRNA transfected versus cells transfected with the I $\kappa$ B $\beta$  expression plasmid alone. Values are means $\pm$ s.e.m.,  $n=3$  per time point. \* $P$ <0.05 versus I $\kappa$ B $\beta$  expression plasmid alone.



**Fig. 4. IκBβ knockdown attenuates LPS-induced expression of select NFκB target genes.** (A) Relative mRNA expression of IκBα and IκBβ in RAW 264.7 macrophages following transfection with IκBβ siRNA. Values are mean±s.e.m.,  $n=9$ . \* $P<0.05$  versus untransfected control. (B) Representative western blot showing IκBα and IκBβ in cytosolic extracts from RAW 264.7 cells following transfection with IκBβ siRNA, with calnexin as the loading control. Densitometric evaluation is provided. (C) Percentage change in LPS-induced gene expression in RAW 264.7 macrophages transfected with IκBβ siRNA. Values are expressed as a ratio of mRNA fold-change in siRNA transfected, LPS-exposed cells versus that in cells exposed to LPS alone. Values are means±s.e.m.,  $n=3$  per time point. \* $P<0.05$  versus LPS-exposed cells.

Cytoplasmic retention of IκB proteins in unstimulated cells, as well as purity of our nuclear fractions is shown in Fig. 5E. Additionally, significant nuclear retention of both cRel and p65 persisted throughout the 4 h exposure in WT MEFs (Fig. 5B,C). Similar to WT MEFs, LPS induced a rapid and robust nuclear translocation of cRel, p65 and p50 in AKBI MEFs (Fig. 5B,C). In contrast to in WT MEFs, after 4 h of exposure there was no significant nuclear retention of any NFκB subunits in AKBI MEFs (Fig. 5B,C). These findings were corroborated by a transcription factor assay that showed by 4 h of LPS exposure only WT MEFs demonstrated significant NFκB DNA binding (Fig. 5D). Taken together, these data demonstrate that the rapid re-accumulation of cytosolic IκBβ following LPS exposure affects the duration of NFκB nuclear translocation.

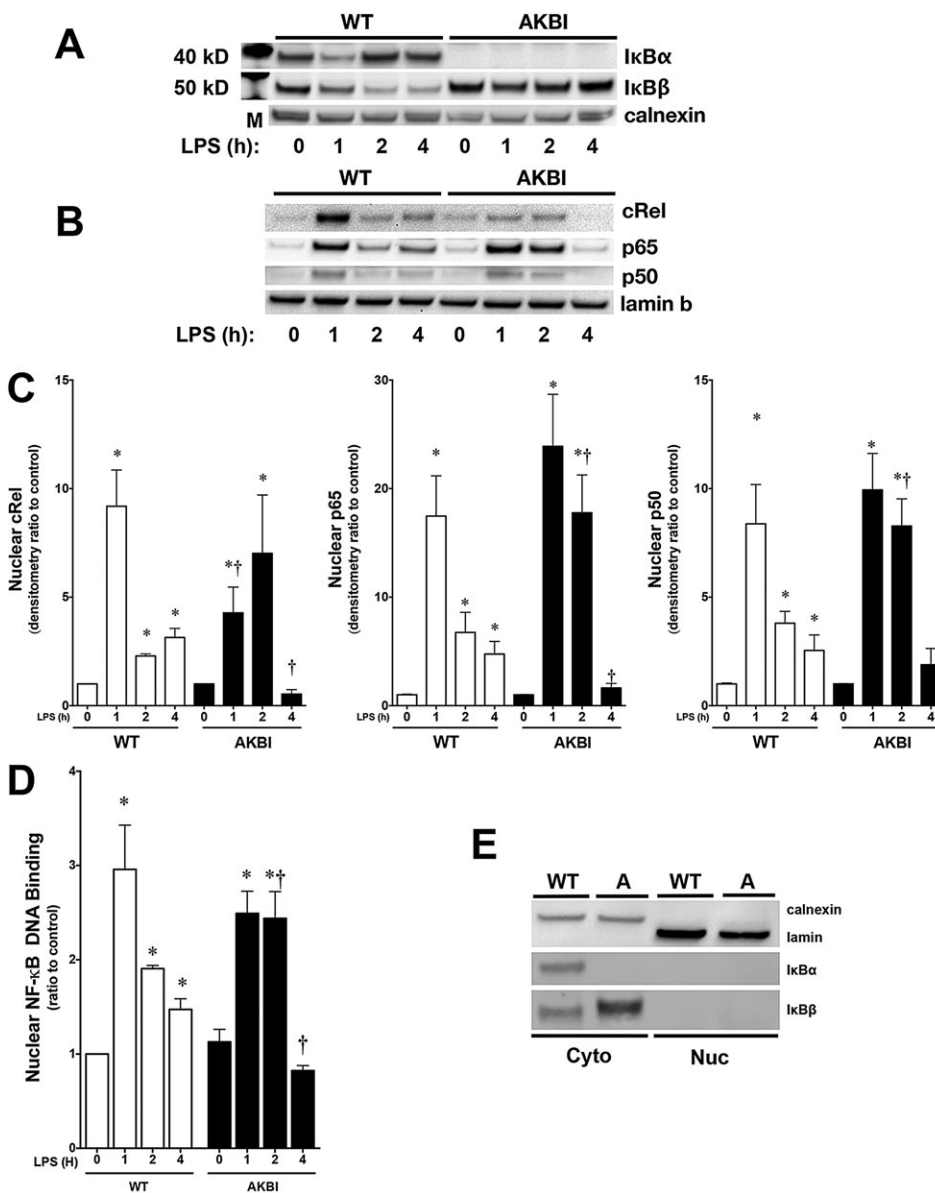
#### IκBβ knock-in MEFs have attenuated LPS-induced expression of target genes sensitive to low-dose pharmacological NFκB blockade

Having observed abbreviated LPS-induced IκBβ–NFκB signaling in AKBI MEFs, we next assessed expression of NFκB target genes. A number of genes were not expressed at baseline or with LPS exposure in either WT or AKBI MEFs (IL1β, IL12β). As anticipated, AKBI MEFs did not express IκBα (Fig. 6B). The pattern of LPS-induced expression of genes not sensitive or less sensitive to low-dose pharmacological inhibition (TNFα, MnSOD, A20, RANTES, CXCL1 and IP-10) was not different between WT and AKBI MEFs (Fig. 6A,B). In contrast, after 5 h LPS exposure, IL6 expression was significantly attenuated in AKBI MEFs when compared to WT, despite no differences being observed after 1 h of exposure (Fig. 6C). To assess the expression of genes not expressed in MEFs (IL1β and IL12β), BMDMs isolated from WT and AKBI mice were exposed to

LPS (1 μg/ml) and assessed for IL1β and IL12β expression (Fig. 6D). Expression of both IL1β and IL12β expression was significantly attenuated in AKBI BMDMs. These results demonstrate that shortened LPS-induced NFκB activation mediated by rapid cytosolic re-accumulation of IκBβ affects the expression of a specific subset of NFκB target genes especially at later time points.

#### IL6 expression is sensitive to IκBβ expression in MEF cells

Next, we sought to confirm that IκBβ–NFκB signaling played a mechanistic role in the LPS-induced IL-6 expression in MEFs. WT MEFs cells transfected with FLAG-tagged IκBβ expression plasmid demonstrated robust IκBβ mRNA and protein expression (Fig. 7A,D). This corresponded with robust induction of IL6 expression in the absence of LPS-activation (Fig. 7B). In contrast, IκBβ overexpression did not affect TNFα and MnSOD expression (Fig. 7B). Furthermore, by titrating the amount of IκBβ expression plasmid used for transfection, a dose-dependent relationship between IκBβ and IL6 expression was demonstrated (Fig. 7C). Similar to RAW 264.7 macrophages, transfection of MEFs with the IκBβ expression plasmid resulted in significant nuclear translocation of both IκBβ and cRel (Fig. 7D,E). Next, we asked whether transfecting AKBI MEFs cells with IκBβ siRNA would return LPS-induced IL-6 expression similar to that observed in similarly exposed WT MEFs. Transfection of IκBβ siRNA resulted in a 30% decrease in immunoreactive IκBβ (Fig. 7F) and mRNA expression (Fig. 7G). With this level of IκBβ knockdown, LPS-induced IL-6 expression significantly increased (>30 fold, Fig. 7H) in AKBI MEFs. These findings show that in AKBI MEFs, rapid cytosolic IκBβ re-accumulation after LPS exposure attenuates IL6 expression, and silencing IκBβ expression is enough to re-establish LPS-induced IL6 expression.



**Fig. 5. AKBI MEFs demonstrate abbreviated LPS-induced NF $\kappa$ B activation.**

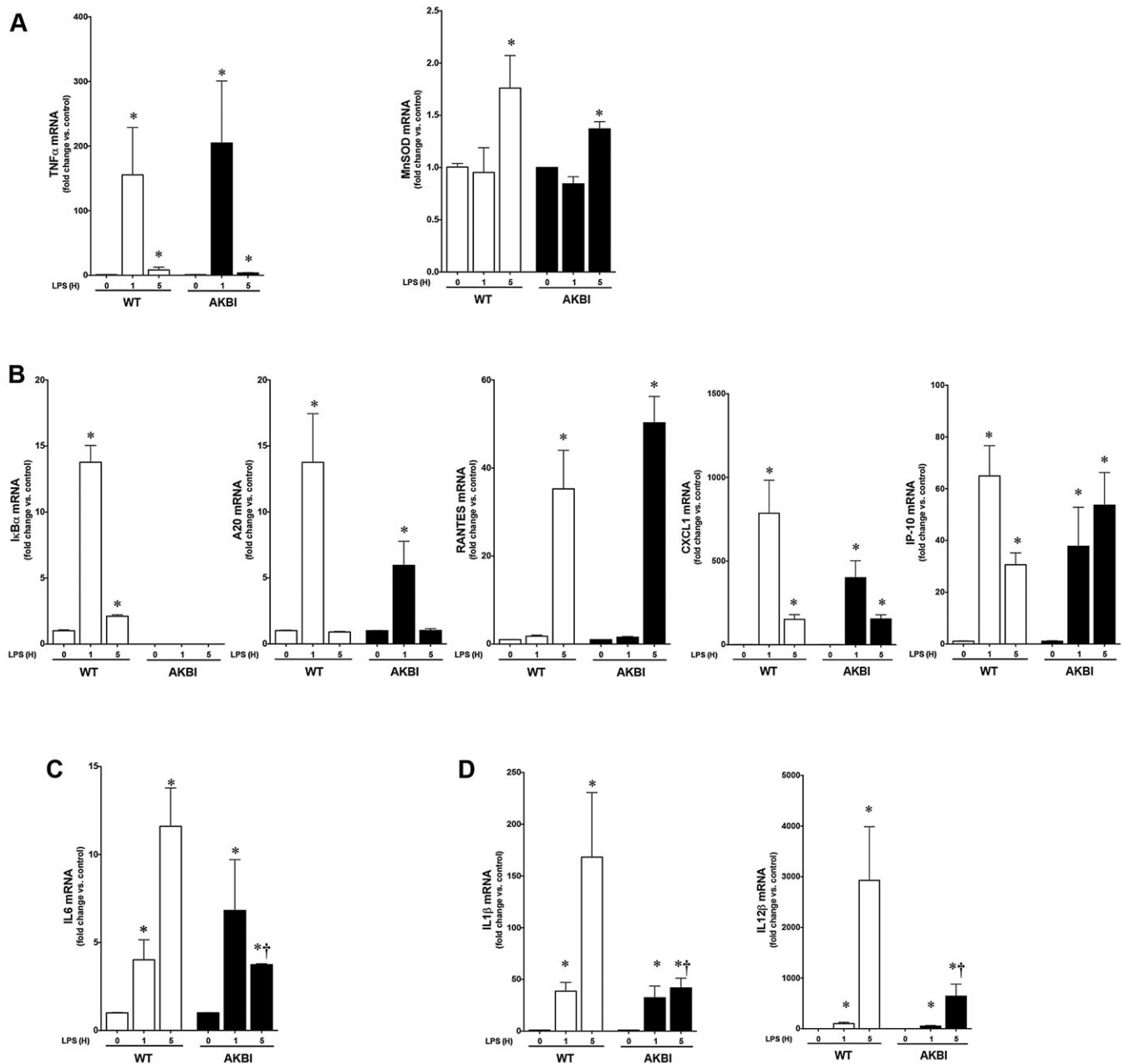
(A) Representative western blot showing cytosolic I $\kappa$ B $\beta$  and I $\kappa$ B $\alpha$  in WT and AKBI MEFs exposed to LPS (1  $\mu$ g/ml, 1–4 h). Calnexin is shown as a loading control. (B) Representative western blot showing nuclear cRel, p65 and p50 in WT and AKBI MEFs exposed to LPS (1  $\mu$ g/ml, 1–4 h). Lamin B is shown as a loading control. (C) Densitometric evaluation of nuclear cRel, p65 and p50 in WT and AKBI MEFs exposed to LPS (1  $\mu$ g/ml, 1–4 h). Results are expressed as a ratio to unexposed control and normalized to the lamin B loading control. Values are mean $\pm$ s.e.m.,  $n=5$  per time point. \* $P<0.05$  versus unexposed control; † $P<0.05$  versus paired WT exposure. (D) Fold change in NF $\kappa$ B DNA binding in WT and AKBI MEFs exposed to LPS as determined by a transcription factor assay. Values are mean $\pm$ s.e.m.,  $n=5$  per time point. \* $P<0.05$  versus unexposed control; † $P<0.05$  versus paired WT exposed. (E) Representative western blot demonstrating purity of cytosolic and nuclear extracts from WT and AKBI MEFs, as well as cytosolic retention of I $\kappa$ B in unstimulated cells. Calnexin was used as a cytosolic marker, whereas lamin B was used as a nuclear marker.

### IL6 expression is sensitive to cRel nuclear translocation in MEF cells

Because I $\kappa$ B $\beta$  overexpression was associated with cRel nuclear translocation and IL6 induction, we sought ways to specifically inhibit cRel nuclear translocation. We designed and synthesized a peptide (SN75) containing the cRel nuclear localization sequence (murine residues 360–368, QRKRKLMP) linked to the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor. Pretreatment of WT MEFs with SN75 (1 h, 25–200  $\mu$ M) attenuated LPS-induced IL6 expression in a dose-dependent manner (Fig. 8A). At a dose that significantly attenuated IL6 expression (100  $\mu$ M, Fig. 8A), SN75 had no effect on LPS-induced TNF $\alpha$  or MnSOD expression (Fig. 8B). Furthermore, examination of nuclear extracts showed that SN75 (200  $\mu$ M) attenuated cRel and p65 nuclear translocation, with no effect on p50 (Fig. 8C). These results suggest that SN75 inhibits LPS-induced nuclear translocation of p65-cRel containing dimers. These results show that inhibiting either cRel or I $\kappa$ B $\beta$  nuclear translocation is sufficient to attenuate LPS-induced IL6 expression.

### DISCUSSION

We found that both pharmacological and genetic manipulations of LPS-induced I $\kappa$ B $\beta$ –NF $\kappa$ B signaling affected the expression of a similar and unique subset of NF $\kappa$ B target genes in macrophages and MEF cells. Using low-dose parthenolide and BAY 11-7085, both well-known and widely used NF $\kappa$ B inhibitors, we show dose-dependent and differential effects on LPS-induced I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  degradation. At low doses, pharmacological NF $\kappa$ B blockade inhibits only I $\kappa$ B $\beta$  degradation, and attenuates the expression of a specific subset of NF $\kappa$ B target genes including those encoding IL6, IL1 $\beta$  and IL12 $\beta$ . Using I $\kappa$ B $\beta$  siRNA and expression plasmids to perform gain- and loss-of-function experiments, we revealed a necessary and sufficient role played by I $\kappa$ B $\beta$  in the expression of these genes. Taken together, these studies demonstrate that both cytosolic degradation of I $\kappa$ B $\beta$  and the nuclear activity of I $\kappa$ B $\beta$  are important aspects of I $\kappa$ B $\beta$ –NF $\kappa$ B signaling. Both events – cytosolic degradation and subsequent nuclear translocation – contribute to the sustained expression of select NF $\kappa$ B target genes. Furthermore, using MEFs and BMDMs derived from WT and I $\kappa$ B $\beta$



**Fig. 6. AKBI MEFs demonstrate attenuated LPS-induced expression of select NF $\kappa$ B target genes.** Fold increase of gene expression of (A) MnSOD and TNF $\alpha$ , (B) I $\kappa$ B $\alpha$ , A20, RANTES, CXCL1 and IP-10, and (C) IL6 following exposure to LPS (1  $\mu$ g/ml, 1 and 5 h) in WT and AKBI MEFs. (D) Fold increase of gene expression of IL1 $\beta$  and IL12 $\beta$  following exposure to LPS (1  $\mu$ g/ml, 1 and 5 h) in WT and AKBI BMDMs. Values are means $\pm$ s.e.m.,  $n=4$  per time point. \* $P<0.05$  versus unexposed control; † $P<0.05$  versus paired WT LPS-exposed cells.

overexpressing mice, we show rapid cytosolic re-accumulation of I $\kappa$ B $\beta$  following LPS abbreviates the duration of NF $\kappa$ B activation, and attenuates the expression of the same target genes that are sensitive to low-dose pharmacological IKK blockade. Further confirming the role of I $\kappa$ B $\beta$  and its preferred NF $\kappa$ B subunit binding partner cRel in LPS-induced gene expression, pre-treatment of WT MEFs with a cell-permeable peptide containing the cRel nuclear localization sequence attenuated cRel nuclear localization and IL6 expression. These results demonstrate the specific role of I $\kappa$ B $\beta$  in mediating the expression of key NF $\kappa$ B target genes following LPS exposure. We prove that LPS-induced NF $\kappa$ B activation can be pharmacologically modulated, rather than completely inhibited, thus affecting the expression of only a subset of target genes. These

findings provide potential therapeutic insights for patients exposed to systemic inflammatory stress.

Here, we report that pharmacological treatment can selectively inhibit LPS-induced, I $\kappa$ B $\beta$ -mediated NF $\kappa$ B activation thereby affecting the expression of specific target genes. These findings have translational implications for future therapies aimed at dampening, rather than completely inhibiting, the innate immune response to inflammation. Pharmacological inhibition of NF $\kappa$ B activation attenuates the expression of pro-inflammatory cytokines and prevents mortality in endotoxemic models of septic shock (Altavilla et al., 2002; Everhart et al., 2006; Fujihara et al., 2000; Liu et al., 1997; Matsuda et al., 2004; Sheehan et al., 2002; Uiloo et al., 2002). Despite this, no therapies that inhibit NF $\kappa$ B activity are



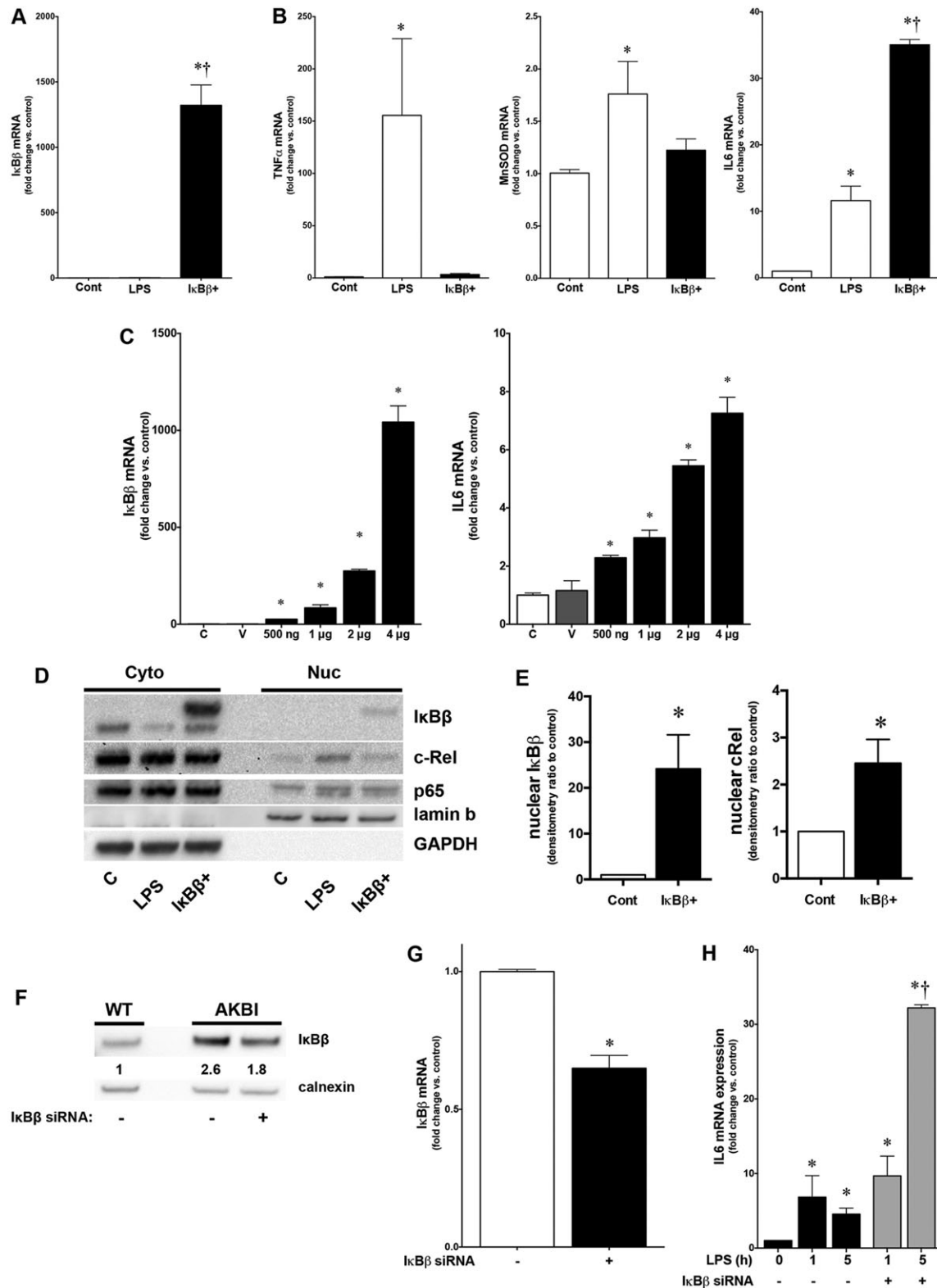


Fig. 7. See next page for legend.

currently used clinically. This can be explained by the fact that completely inhibiting NFκB activity is not without risk and can worsen outcomes in preclinical models (Courtine et al., 2011; Gadjeva et al., 2004; Greten et al., 2007; Kisseleva et al., 2006; Lawrence et al., 2005; Sha et al., 1995). Thus, identifying strategies

to attenuate the NFκB response must be identified and evaluated to determine whether they have potential therapeutic effect in patients with septic shock (Rahman and Fazal, 2011; Wheeler et al., 2009).

Previous studies have shown that IκBα and IκBβ confer specificity to the NFκB transcriptome in response to inflammatory stress. The

**Fig. 7. I $\kappa$ B $\beta$  overexpression causes cRel dependent expression of NF $\kappa$ B target genes in MEFs.** (A) Relative I $\kappa$ B $\beta$  mRNA expression in WT MEFs following transfection with FLAG-tagged I $\kappa$ B $\beta$  expression plasmid. Values are mean $\pm$ s.e.m.,  $n=6$ . \* $P<0.05$  versus untransfected control;  $^{\dagger}P<0.05$  versus LPS-exposed cells. (B) Relative TNF $\alpha$ , MnSOD and IL-6 mRNA expression in WT MEFs following transfection with I $\kappa$ B $\beta$  expression plasmid (I $\kappa$ B $\beta$ +). Values are mean $\pm$ s.e.m.,  $n=6$ . \* $P<0.05$  versus untransfected control;  $^{\dagger}P<0.05$  versus LPS-exposed cells. (C) Relative I $\kappa$ B $\beta$  and IL-6 mRNA expression in WT MEFs following transfection with increasing amounts of I $\kappa$ B $\beta$  expression plasmid ranging from 500 ng to 4  $\mu$ g per transfection. C, untransfected control; V, vehicle alone. Values are mean $\pm$ s.e.m.,  $n=3$ . \* $P<0.05$  versus untransfected control. (D) Representative western blot showing I $\kappa$ B $\beta$ , cRel, and p65 in cytosolic and nuclear extracts from WT MEFs either exposed to LPS (1  $\mu$ g/ml, 4 h) or transfected with I $\kappa$ B $\beta$  expression plasmid (I $\kappa$ B $\beta$ +), with GAPDH as the cytosolic loading control and lamin B as the nuclear loading control. (E) Densitometric evaluation of nuclear I $\kappa$ B $\beta$  and cRel in WT MEFs (cont) or transfected with I $\kappa$ B $\beta$  expression plasmid (I $\kappa$ B $\beta$ +). Results are expressed as a ratio to unexposed control and normalized to the lamin B loading control. Values are mean $\pm$ s.e.m.,  $n=3$ . \* $P<0.05$  versus non-transfected control. (F) Representative western blot showing I $\kappa$ B $\beta$  in cytosolic extracts from WT MEFs, AKBI MEFs and AKBI MEFs cells following transfection with I $\kappa$ B $\beta$  siRNA, with calnexin as the loading control. A densitometric evaluation is provided. (G) Relative I $\kappa$ B $\beta$  mRNA expression in AKBI MEFs following transfection with I $\kappa$ B $\beta$  siRNA. Values are mean $\pm$ s.e.m.,  $n=6$ . \* $P<0.05$  versus untransfected control. (H) Relative LPS-induced (1  $\mu$ g/ml, 1 and 5 h) IL6 mRNA expression in AKBI MEFs following transfection with I $\kappa$ B $\beta$  siRNA. Values are mean $\pm$ s.e.m.,  $n=6$ . \* $P<0.05$  versus unexposed control;  $^{\dagger}P<0.05$  versus cells exposed to LPS alone.

penultimate step of NF $\kappa$ B nuclear translocation and DNA binding converges on the inhibitory proteins I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  (Hayden and Ghosh, 2004). In quiescent cells, I $\kappa$ B proteins sequester NF $\kappa$ B in the cytoplasm (Hayden and Ghosh, 2004). Exposure to LPS results in phosphorylation and degradation of these proteins, allowing NF $\kappa$ B nuclear translocation (Hayden and Ghosh, 2004). Although early studies described I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  as ‘functionally redundant’ (Cheng et al., 1998), I $\kappa$ B $\beta$  was also found to have a unique role in mediating the ‘persistent’ inflammatory stress-induced response of NF $\kappa$ B (Thompson et al., 1995). Owing to structural differences, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  preferentially bind to unique NF $\kappa$ B dimer pairs, with I $\kappa$ B $\beta$  preferentially binding to cRel-containing dimers (Hoffmann et al., 2002; Thompson et al., 1995). Thus, upon degradation, unique NF $\kappa$ B dimer pairs translocate to the nucleus. Additionally, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  have unique nuclear activity following re-synthesis after degradation. Following degradation, both newly synthesized I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  enter the nucleus. A nuclear export sequence found on I $\kappa$ B $\alpha$  allows it to remove DNA-bound NF $\kappa$ B complexes from the nucleus (Hayden and Ghosh, 2004). In contrast, I $\kappa$ B $\beta$  lacks a nuclear export sequence (Hayden and Ghosh, 2004), and remains in the nucleus and facilitates the ‘late-phase’ of the NF $\kappa$ B response by stabilizing DNA binding of cRel-containing NF $\kappa$ B dimers (Chu et al., 1996; Suyang et al., 1996; Thompson et al., 1995; Thompson et al., 1995; Tran et al., 1997). Importantly, cRel containing dimers target a very select subset of NF $\kappa$ B target genes (Sen and Smale, 2010).

Interestingly, we could find no reports evaluating the effect of pharmacological inhibitors of NF $\kappa$ B activity on I $\kappa$ B $\beta$ –NF $\kappa$ B signaling following LPS-exposure or endotoxemic shock. Recently, two separate laboratories have demonstrated that I $\kappa$ B $\beta$ <sup>-/-</sup> mice are extremely resistant to endotoxemic shock, a finding explained by attenuated expression of key pro-inflammatory NF $\kappa$ B target genes including IL6, IL1 $\beta$  and IL12 $\beta$  (Rao et al., 2010; Scheibel et al., 2010). Adding further mechanistic insight into these findings, cRel was found play a crucial role in the sustained expression of these genes. In what appears to conflict with these findings, cRel<sup>-/-</sup> mice show increased sensitivity to polymicrobial sepsis (Courtine et al., 2011). However, cRel<sup>-/-</sup> mice have impaired

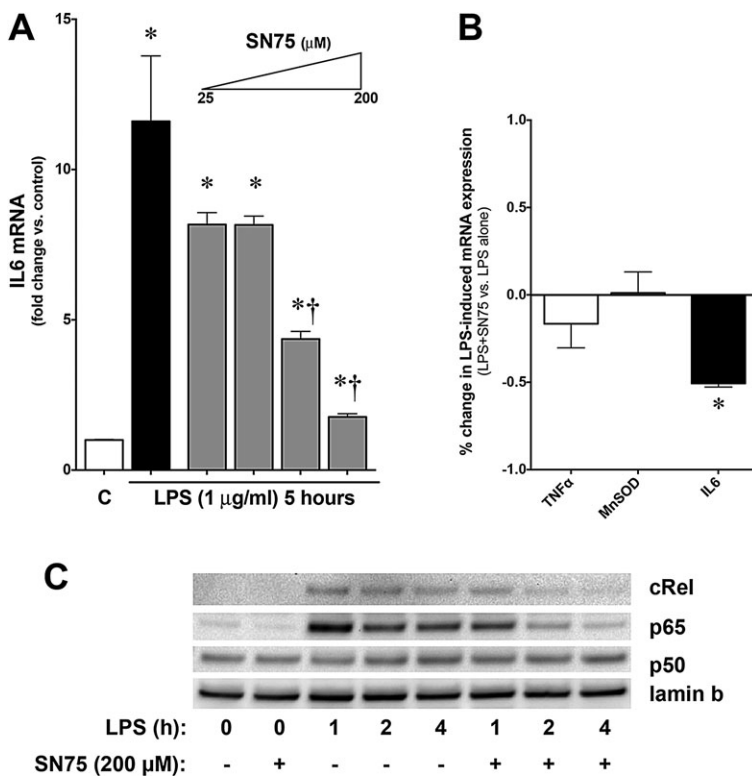
lymphoid dendritic cell development, complicating the interpretation of these findings. Thus, the insights gained in the current study using genetic modifications of I $\kappa$ B $\beta$  and cRel inform our understanding of the subtleties underlying the NF $\kappa$ B response to inflammatory stress.

Multiple pharmacological inhibitors of NF $\kappa$ B signaling have been identified (Gilmore and Herscovitch, 2006). Both BAY 11-7085 and parthenolide have been identified as IKK inhibitors (Gilmore and Herscovitch, 2006). The original report describing BAY 11-7085 as an NF $\kappa$ B inhibitor demonstrated that a dose of 20  $\mu$ M was sufficient to inhibit I $\kappa$ B $\alpha$  degradation (Pierce et al., 1997). Importantly, the effect on I $\kappa$ B $\beta$  degradation was not assessed. Since this report, these agents have become so widely accepted as NF $\kappa$ B inhibitors that their effect on I $\kappa$ B $\alpha$  degradation is frequently not reported. Scattered data suggests that there is a dose-dependent effect of these inhibitors on NF $\kappa$ B signaling. Parthenolide pretreatment at doses >10  $\mu$ M inhibits LPS-induced I $\kappa$ B $\alpha$  degradation (Yip et al., 2004), p65 nuclear translocation (Li et al., 2006) and NF $\kappa$ B reporter activity in RAW 264.7 cells (Park et al., 2011). Interestingly, parthenolide has been reported to attenuate LPS-induced expression of NF $\kappa$ B target genes in a dose-dependent manner (at doses of 0.4–4  $\mu$ M) lower than those previously demonstrated to inhibit I $\kappa$ B $\alpha$  degradation (De Silva et al., 2010; Magni et al., 2012). However, the mechanism underlying this finding has not been determined. We have previously reported that pre-treating fetal pulmonary endothelial cells with low-dose parthenolide specifically attenuates LPS-induced I $\kappa$ B $\beta$  degradation (Tang et al., 2013). In the current report, we expand upon those findings adding mechanistic insights. To our knowledge, this is the first report demonstrating that LPS-induced, I $\kappa$ B $\beta$ -mediated NF $\kappa$ B activation and gene expression can be pharmacologically targeted.

The observation that low-dose pharmacological inhibition of IKK specifically attenuates I $\kappa$ B $\beta$  degradation could be explained by the affinity of the IKK complex for I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ . Previous reports indicate that the kinetics of I $\kappa$ B $\beta$  degradation is unique and slower than that of I $\kappa$ B $\alpha$  following LPS stimulation (Thompson et al., 1995). Additionally, IKK is known to have a lower affinity for I $\kappa$ B $\beta$  when compared to I $\kappa$ B $\alpha$  (Heilker et al., 1999; Mercurio et al., 1997). It is possible that at lower doses, both parthenolide and BAY 11-7085 inhibit IKK activity enough to prevent phosphorylation of proteins that interact with IKK with lower affinity, including I $\kappa$ B $\beta$ . These hypotheses remain to be tested and their validity confirmed.

The current study has a number of limitations. We evaluated only a very select subset of known NF $\kappa$ B target genes. However, we chose a diverse set of LPS-induced, pro-inflammatory NF $\kappa$ B targets that were either MyD88 independent (IP10, RANTES) or MyD88 dependent (IL1 $\beta$ , CXCL1, TNF, IL12 $\beta$ , IL6). (Björkbacka et al., 2004). Additionally, NF $\kappa$ B target genes that regulate NF $\kappa$ B activity (I $\kappa$ B $\alpha$ , A20) and antioxidant enzymes (MnSOD) were assessed (Gilmore and Herscovitch, 2006; Verstrepen et al., 2010). Undoubtedly, LPS-induced expression of other NF $\kappa$ B target genes will also demonstrate variable sensitivity to low-dose parthenolide and BAY 11-7085. These target genes remain to be identified using larger, genome-wide explorations. Our data provide justification for further study of this important question.

Additionally, the use of pharmacological agents can be complicated by off-target effects. For example, parthenolide can affect MAP kinase, ERK and adaptor protein complex 1 (AP-1) activity (Fiebich et al., 2002; Hwang et al., 1996; Saadane et al., 2011). This raises the possibility that our findings were due to mechanisms other than inhibition of LPS-induced, I $\kappa$ B $\beta$ -mediated NF $\kappa$ B activation.



**Fig. 8. Inhibition of LPS-induced cRel nuclear translocation attenuates IL6 expression.** (A) Relative IL6 mRNA expression in WT MEFs following exposure to LPS (1 μg/ml, 5 h) or following pre-treatment with a cell-permeable synthetic peptide containing the cRel nuclear localization sequence (SN75, 25–200 μM) prior to exposure to LPS (1 μg/ml, 5 h). Values are mean±s.e.m.,  $n=6$ . \* $P<0.05$  versus untransfected control; † $P<0.05$  versus LPS-exposed cells. (B) Percentage change in LPS-induced (1 μg/ml, 5 h) TNFα, MnSOD and IL6 expression in WT MEFs following pre-treatment with SN75 (100 μM, 1 h). Values are expressed as a ratio of mRNA fold change in LPS-exposed cells with SN75 pretreatment versus cells with LPS exposure alone. Values are mean±s.e.m.,  $n=3$  per time point. \* $P<0.05$  versus IκBβ expression plasmid alone. (C) Representative western blot showing cRel, p65 and p50 in nuclear extracts from WT MEFs either exposed to LPS (1 μg/ml, 5 h) or following pre-treatment with a cell-permeable synthetic peptide containing the cRel nuclear localization sequence (SN75, 200 μM) prior to exposure to LPS (1 μg/ml, 5 h) with lamin B as the nuclear loading control.

Therefore, we sought to confirm the effects of low-dose IKK inhibition on LPS-induced target gene expression by genetically modifying IκBβ-mediated NFκB signaling. The LPS-induced expression of IL6, IL1β and IL12β was significantly attenuated, by >50%, upon silencing IκBβ expression, and robustly induced by overexpression of IκBβ in the absence of LPS activation. Additionally, the effect of IκBβ overexpression on IL6, IL1β and IL12β expression was significantly attenuated by co-transfection with cRel siRNA. It is important to note that silencing IκBβ expression did not completely attenuate LPS-induced expression of these target genes, and silencing cRel expression did not completely attenuate expression of these target genes induced by IκBβ overexpression. Therefore, other redundant mechanisms regulating gene expression are likely present.

Supporting these data, LPS-induced expression of IL6, IL1β and IL12β are significantly attenuated in MEFs and BMDMs isolated from IκBβ overexpressing mice, secondary to attenuated nuclear translocation of NFκB. Of note, baseline expression of IL6, IL1β and IL12β was not higher in AKBI cells compared to WT cells. These findings are in contrast to what was observed with IκBβ overexpression induced by transfection. These differences are likely due to the level of IκBβ overexpression (3-fold in AKBI versus 2000–3000-fold after transfection). Furthermore, baseline levels of nuclear cRel, p65 and p50 were not different between unstimulated WT and AKBI cells, further confirming that nuclear translocation of both IκBβ and NFκB subunits was responsible for the induced expression of IL6, IL1β and IL12β. These data confirm that a mechanistic role is played by nuclear IκBβ and cRel in the expression of select NFκB target genes, and that this pathway can be pharmacologically targeted. However, development and evaluation of more selective inhibitors of IκBβ–NFκB signaling are necessary to confirm that this pathway can be pharmacologically targeted. Furthermore, we did not perform chromatin immunoprecipitation on the promoter regions of the IL6-, IL1β- and IL12β-encoding genes and thus

cannot rule out the presence of other important transcription factors that contribute to their expression. Finally, we performed experiments in macrophages and MEF cells. It is known that NFκB signaling is cell type specific, and it is unknown whether these findings are pertinent to additional cell types.

We conclude that expression of pro-inflammatory mediators induced by LPS exposure can be manipulated by targeting IκBβ–NFκB signaling. This robust analysis of the effect of low-dose pharmacological NFκB blockade, complimented by genetic modification of IκBβ–NFκB signaling, supports our hypothesis that specifically targeting IκBβ–NFκB signaling attenuates the expression of a select subset of NFκB target genes. These mechanistic insights provide evidence that supports further study of interventions targeting IκBβ–NFκB signaling as a way to dampen – rather than completely inhibit – the innate immune response to inflammatory stress. These findings may inform future strategies to improve the outcomes of patients exposed to systemic inflammatory stress.

## MATERIALS AND METHODS

### Cell culture, LPS exposure and pharmacological NFκB inhibition

RAW 264.7 murine macrophages (ATCC) were cultured according to the manufacturer's instructions. ICR and AKBI MEFs were cultured as described previously (Wright et al., 2012). BMDMs were collected from 6–10-week-old male ICR mice and were cultured as previously described (Zhang et al., 2008). Cells were exposed to LPS (1 μg/ml, 0555:B5, Sigma). For pharmacological inhibition studies, cells were pretreated with NFκB inhibitors BAY 11-7085 (Sigma-Aldrich) or Parthenolide (Sigma-Aldrich) at varying doses for 1 h prior to LPS exposure; inhibitors were maintained in the culture media throughout the LPS exposure.

### Transfection with IκBα or IκBβ overexpression plasmids, and IκBβ, cRel and p65 siRNA

To establish IκBβ overexpression, RAW 264.7 and ICR MEFs cells were grown to 70% confluence and transfected with a flag-tagged IκBβ vector (Genecopoeia, catalog number Ex-Mm04103-M12) or pCMV-IκBα vector



(Clontech) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To achieve I $\kappa$ B $\beta$ , cRel and p65 silencing, cells were transfected with siRNA (Dharmacon catalog numbers: I $\kappa$ B $\beta$  L-047122-00-0005; cRel L-061277-01-0005; p65 L-040776-00) using Dharmafect Duo according to the manufacturer's instructions.

### Cytosolic and nuclear extraction and mRNA isolation

Cytosolic and nuclear extracts were collected using the NE-PER Kit (Pierce) according to the manufacturer's instructions. Alternatively, cells were washed in ice-cold PBS and lysed in Buffer RLT (Qiagen). RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was assessed for purity and concentration using the Nanodrop (ThermoFisher Scientific) and cDNA was synthesized using the Verso cDNA Kit (Thermo Scientific).

### Analysis of relative mRNA levels by qRT-PCR

Relative mRNA levels were evaluated by quantitative real-time PCR using the TaqMan gene expression system (Applied Biosystems). Gene expression was assessed using pre-designed, species-appropriate exon-spanning primers (catalog numbers are as follows: MnSOD, Mm00449726\_m1; TNF, Mm00443258\_m1; I $\kappa$ B $\alpha$ , Mm00477798\_m1; A20, Mm00437121\_m1; RANTES, Mm01302428\_m1; CXCL1, Mm04207460\_m1; IP10, Mm00445235\_m1; IL1 $\beta$ , Mm01336189\_m1; IL6, Mm00446190\_m1; IL12 $\beta$ , Mm00434174\_m1) using the StepOnePlus Real Time PCR System (Applied Biosystems). Relative quantification was performed by normalization to the endogenous control 18S RNA using the cycle threshold ( $\Delta\Delta C_t$ ) method.

### Immunoblot analysis

Cytosolic and nuclear extracts were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen) and proteins were transferred to an Immobilon membrane (Millipore). Membranes were blotted with antibodies against I $\kappa$ K $\alpha$  (Cell Signaling 2682), IKK $\beta$  (Cell Signaling 2370), IKK $\epsilon$  (Cell Signaling 3416), I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology 371), I $\kappa$ B $\beta$  (Santa Cruz Biotechnology 9130), p65 (Abcam 7970), p50 (Abcam 7971), cRel (Cell Signaling 4727), Calnexin (Enzo Life Sciences ADI-SPA-860) and Lamin B (Santa Cruz Biotechnology 6216). Densitometric analysis was performed using ImageLab (Bio-Rad).

### Evaluation of nuclear NF $\kappa$ B binding by a transcription factor assay

A NF $\kappa$ B-specific transcription factor assay was performed according to the manufacturer's instructions (catalog number 70-510, Upstate). Briefly, 5 mg of nuclear extract was incubated with biotinylated double-stranded NF $\kappa$ B consensus sequence oligonucleotides in a 96-well streptavidin-coated plate. After washing, antibody against the p65 and p50 subunits was added to the wells and allowed to incubate overnight. After washing, anti-rabbit-IgG secondary antibody was added to the wells and allowed to incubate for 40 min. Wells were washed, incubated with TMB/E for 10 min, and absorbance of the samples was performed using a spectrophotometric microplate reader set at 1450 nm.

### cRel nuclear translocation inhibition

To inhibit cRel nuclear translocation, ICR MEFs were treated with synthetic peptide SN75 containing the cRel nuclear localization sequence (murine residues 360–368, QRKRKLM) linked to the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor. SN75 was synthesized by the University of Colorado Peptide, Protein Chemistry Core (Aurora, CO). Peptide purity (>98%) validation and molecular mass determination was performed by the University of Colorado Denver Peptide/Protein Chemistry Core using an Agilent 1100 LC/MS. ICR MEFs were pretreated with 100  $\mu$ g/ml or 200  $\mu$ g/ml SN75 for 1 h prior to LPS exposure; SN75 was maintained in the culture media during the exposure period.

### Statistical analysis

For comparison between treatment groups, the null hypothesis that no difference existed between treatment means was tested by Student's *t*-test for two groups and two-way ANOVA for multiple groups with potentially

interacting variables (genotype or LPS exposure), with statistical significance between and within groups determined by means of the Bonferroni method of multiple comparisons (InStat, GraphPad Software, Inc). Statistical significance was defined as  $P < 0.05$ .

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

S.M. participated in the design, execution and interpretation of the findings being published, as well as the drafting and the revision of the article. C.J.W. participated in the conception, design, execution and interpretation of the findings being published, as well as the drafting and the revision of the manuscript.

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### Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.168351/-/DC1>

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