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RESEARCH ARTICLE

Inhibiting IκBβ–NFκB 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 κB (NFκB). However, complete NFκB inhibition can exacerbate disease, necessitating evaluation of targeted strategies to attenuate the pro-inflammatory response. Here, we demonstrate that in murine macrophages, low-dose NFκB inhibitors specifically attenuates lipopolysaccharide (LPS)-induced IκBβ degradation and the expression of a select subset of target genes (encoding IL1β, IL6, IL12β). Gain- and loss-of-function experiments demonstrate the necessary and sufficient role of inhibitor of NFκB family member IκBβ (also known as NFKBIB) in the expression of these genes. Furthermore, both fibroblasts and macrophages isolated from IκBβ overexpressing mice demonstrate attenuated LPS-induced IκBβ–NFκB signaling and IL1β, IL6 and IL12β expression. Further confirming the role of IκBβ and its NFκB 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 IκBβ–NFκB 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: NFκB, IκB 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 κB (NFκB) has been implicated in the pathogenesis of septic shock (Liu and Malik, 2006). As such, NFκB 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 NFκB 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 NFκB activity would have both beneficial and detrimental consequences. However, it remains unclear whether the NFκB 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 NFκB transcriptional response to inflammatory stimuli. Understanding these factors might reveal therapeutic targets to attenuate – rather than completely inhibit – NFκB activity. The key step in canonical NFκB activation induced by inflammatory stimuli is phosphorylation and proteolysis of the inhibitor of NFκB (IκB) family of inhibitory proteins. In quiescent cells, members of the IκB family of NFκB inhibitory proteins, IκBα, IκBβ and IκBε, keep inactivated NFκB dimers in the cytoplasm (Hayden and Ghosh, 2004). Following exposure to inflammatory stimuli [e.g. lipopolysaccharide (LPS)], the IκBs are degraded, allowing NFκB nuclear translocation (Hayden and Ghosh, 2004). Although all cytoplasmic IκBs inhibit NFκB activation, IκBβ plays a unique role in determining specific target gene expression. Because IκBβ preferentially binds cRel-containing NFκB dimers, and these dimer combinations bind to unique DNA sequences, specific downstream genes are targeted (Sen and Smale, 2010). Additionally, following degradation, both IκBα and IκBβ are resynthesized and enter the nucleus. A nuclear export sequence (NES) found on IκBα allows it to export DNA-bound NFκB complexes from the nucleus (Hayden and Ghosh, 2004). In contrast, IκBβ lacks a NES, (Tam and Sen, 2001) and remains in the nucleus to stabilize NFκB–DNA binding (Sen and Smale, 2010). Thus, it is possible that targeting IκBβ-mediated NFκB activation could attenuate the persistent expression of specific target genes implicated in the pathogenesis of septic shock.

Studies in *Nfkbib*$^{-/-}$ (hereafter IκBβ$^{-/-}$) mice support this hypothesis. When compared to wild-type (WT) mice exposed to a lethal dose of intraperitoneal LPS, IκBβ$^{-/-}$ mice show improved survival secondary to attenuated pro-inflammatory gene expression (encoding TNFα, IL1β and IL6) (Rao et al., 2010; Scheibel et al., 2010). Despite this, specific pharmacological inhibitors of IκBβ–NFκB have not been reported. Recently, we observed that pretreating fetal pulmonary endothelial cells with a low dose of parthenolide, a known NFκB inhibitor, attenuated LPS-induced IκBβ degradation, while not affecting IκBα degradation (Tang et al., 2013). Importantly, how specifically attenuating LPS-induced IκBβ degradation affects target gene expression is unknown.

Therefore, we hypothesized that inhibition of LPS-induced IκBβ degradation would result in attenuated expression of select NFκB target genes. We found that low-dose NFκB inhibitors (BAY 11-7085 and parthenolide) attenuated only LPS-induced IκBβ degradation in RAW 264.7 macrophages. Analysis of LPS-induced NFκB target gene expression revealed that a subset of genes (encoding IL1β, IL6 and IL12β) were exquisitely sensitive to low-dose NFκB inhibitors. We next genetically modified IκBβ expression to confirm its mechanistic role in the expression of these specific NFκB target genes. Surprisingly, IκBβ 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 IκBβ resulted in IκBβ–cRel nuclear translocation. Further implicating IκBβ–NFκB signaling in the expression of select

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NFκB target genes, silencing IκBβ expression prior to LPS exposure significantly attenuated IL1β, IL6 and IL12β expression. Additionally, LPS-induced gene expression was assessed in mouse embryonic fibroblasts (MEFs) and bone-marrow-derived macrophages (BMDMs) isolated from IκBβ overexpressing (AKBI) mice. Consistent with cytoplasmic IκBβ overexpression, AKBI cells demonstrated abbreviated LPS-induced IκBβ–NFκB signaling and had attenuated IL1β, IL6 and IL12β expression. Further confirming a mechanistic role for IκBβ and its NFκB 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 IκBβ–NFκB signaling attenuates expression of a specific subset of NFκB 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 NFκB inhibitors BAY 11-7085 and parthenolide have dose-dependent effects on LPS-induced IκB degradation

We have previously reported that in fetal pulmonary artery endothelial cells low-dose parthenolide inhibits LPS-induced IκBβ degradation while not affecting IκBα 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 μM, 1 h) or parthenolide (1–5 μM, 1 h) prior to LPS stimulation (30 min, 1 μg/ml). At these doses, neither BAY 11-7085 nor parthenolide prevented LPS-induced IκBα degradation (Fig. 1A). In contrast, beginning at 1 μM, both BAY 11-7085 and parthenolide attenuated IκBβ degradation (Fig. 1A). Consistent with previous reports, higher doses (10–20 μM) of both BAY 11-7085 and parthenolide prevented IκBα and IκBβ degradation (Fig. 1B). These data demonstrated that LPS-induced IκBβ degradation is more sensitive than IκBα to pharmacological inhibitory κB kinase (IKK) inhibition. Selective inhibition of LPS-induced IκBβ degradation attenuated, but did not abolish, NFκB [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 IκBβ degradation. Furthermore, specifically inhibiting IκBβ–NFκB signaling attenuates but does not completely inhibit nuclear translocation of NFκB subunits.

Low-dose BAY 11-7085 and parthenolide attenuate LPS-induced expression of select NFκB target genes

Next, we evaluated the effect of low-dose NFκB 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 IL1β, CXCL1, TNFα, IL12β, IL6) NFκB-dependent target genes were assessed (Björkbacka et al., 2004). Additionally, target genes that regulate NFκB activity [encoding IκBα, 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 NFκB inhibition. Low-dose parthenolide or BAY 11-7085 (1–5 μM) did not affect LPS-induced expression of MnSOD and TNFα (Fig. 2A). In contrast, both BAY 11-7085 and parthenolide significantly attenuated LPS-induced expression of IκBα, A20, RANTES, CXCL1 and IP-10 beginning at 5 μM (Fig. 2B). Finally, LPS-induced expression of IL1β, IL6 and IL12β was extremely sensitive to the lowest dose of BAY 11-7085 and parthenolide tested (1 μM, Fig. 2C). Of note, both parthenolide and BAY 11-7085 at this dose inhibited LPS-induced IκBβ degradation, without affecting IκBα degradation (Fig. 1A–C). Additionally, this dose of parthenolide only attenuates, but does not prevent NFκB nuclear translocation (Fig. 1D). These results suggest that IκBβ degradation is required for LPS-induced expression of a subset of NFκB target genes, and that this pathway can be pharmacologically manipulated to affect the expression of specific NFκB target genes.

Nuclear IκBβ induces expression of target genes sensitive to low-dose pharmacologic NFκB blockade

It is possible that unrecognized, off-target effects of low-dose pharmacological NFκB inhibitors affected IL1β, IL6 and IL12β expression independently of IκBβ (Fig. 2C). Thus, we modified IκBβ expression in RAW 264.7 cells to define its mechanistic role in IL1β, IL6 and IL12β expression. Cells transfected with FLAG-tagged IκBβ expression plasmid demonstrated robust induction of IκBβ mRNA expression (Fig. 3A). Surprisingly, IκBβ overexpression – even in the absence of LPS activation – induced a robust transcriptional response of genes most sensitive to low-dose NFκB inhibition (IL6, IL1β and IL12β) (Fig. 3B). This effect was not present with IκBα overexpression induced by transfection (supplementary material Fig. S1A). Of note, in the setting of transient IκBβ overexpression, LPS-induced expression of these genes was significantly attenuated, suggesting a common mechanism underlying LPS- and IκBβ-induced expression of these specific target genes (supplementary material Fig. S1B). In contrast to the effect observed on IL6, IL1β and IL12β, IκBβ overexpression had a variable effect on the expression of other target genes assessed (MnSOD, TNFα, IκBα, A20, RANTES, CXCL1, IP-10; Fig. 3B). Although cytoplasmic IκBβ is classically considered an inhibitory protein, nuclear IκBβ enhances gene expression (Rao et al., 2010). With this in mind, we sought to define the mechanisms underlying IκBβ-induced expression of select NFκB target genes. Western blot analysis confirmed that transfection with the IκBβ expression plasmid resulted in increased IκBβ protein expression (Fig. 3C, lane 2). Additionally, transfection alone was associated with nuclear translocation of IκBβ, cRel and p65 (Fig. 3C, lane 4). Of note, IκBβ overexpression modestly induced cRel, but not p65, expression (supplementary material Fig. S2). To determine whether cRel played a role in IκBβ-induced gene expression, RAW 264.7 cells were co-transfected with IκBβ expression plasmid and cRel small interfering RNA (siRNA) to achieve >50% reduction in cRel expression (Fig. 3D). Furthermore, macrophages co-transfected with the IκBβ expression plasmid and cRel siRNA demonstrated a ∼50% decrease in IL1β, IL6 and IL12β expression when compared to IκBβ-transfection alone (Fig. 3E; unadjusted data available in supplementary material Fig. S3). Interestingly, similar levels of p65 knock-down did not significantly affect IκBβ-induced expression of these genes (Fig. 3F,G). These data demonstrate that IκBβ overexpression induces IL1β, IL6, IL12β expression through a cRel dependent mechanism.

Silencing IκBβ expression attenuates LPS-induced expression of target genes sensitive to low-dose pharmacological NFκB blockade

Having demonstrated that IκBβ overexpression induces IL1β, IL6 and IL12β, we sought to assess the effect of silencing IκBβ expression on LPS-induced gene expression. We reasoned that following cytosolic IκBβ degradation, re-accumulation and nuclear translocation of IκBβ would play mechanistic role in the LPS-induced expression of IL6, IL1β and IL12β. Thus, silencing IκBβ

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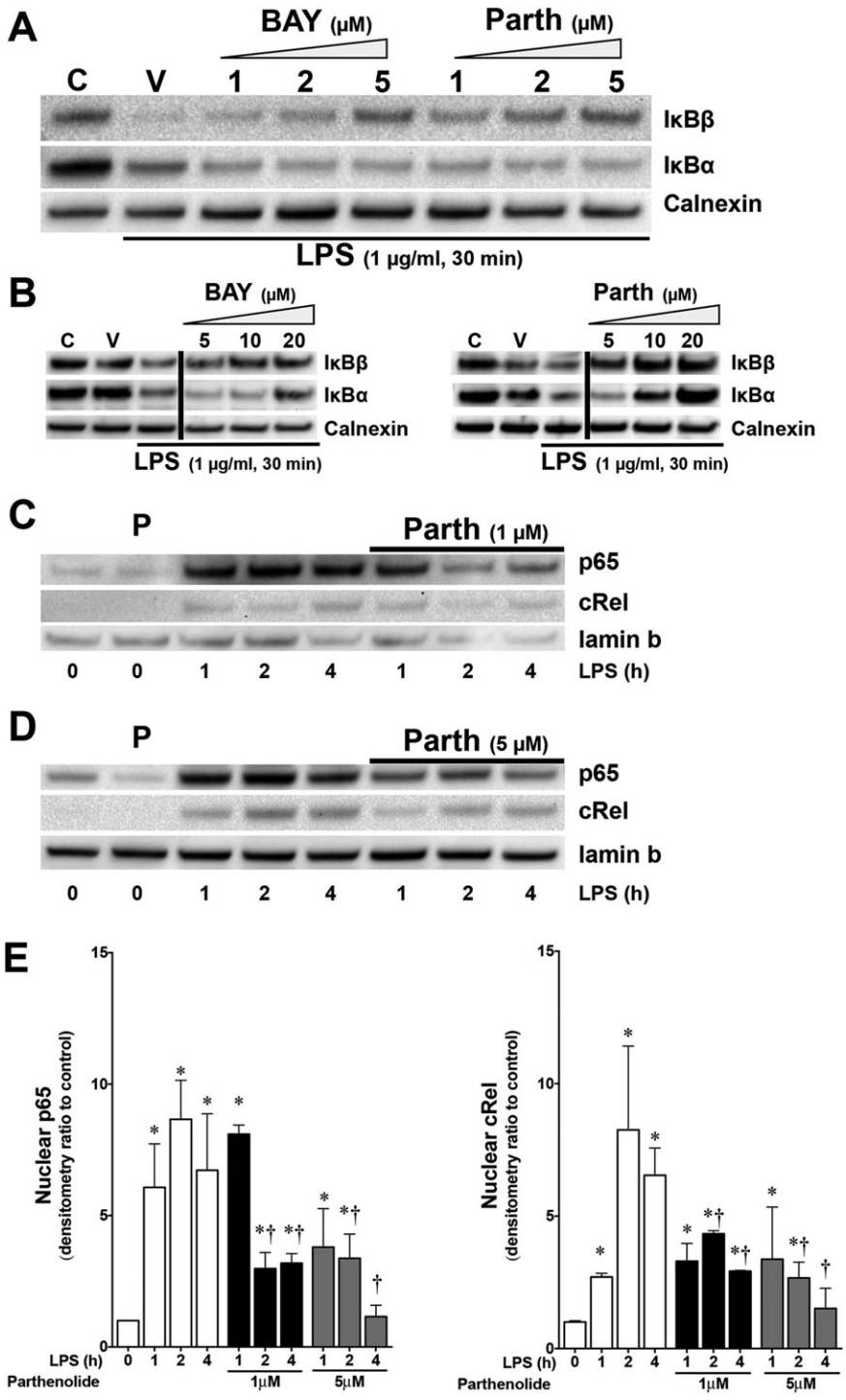

Fig. 1. Pharmacological NFκB inhibitors have dose-dependent effects on LPS-induced IκB degradation in RAW 264.7 macrophages. (A) Representative western blot showing IκBα and IκBβ in cytosolic fractions following exposure to LPS (30 min), or LPS after pretreatment (1 h) with BAY 11-7085 (1–5 μM) or parthenolide (1–5 μM) with calnexin as the loading control. (B) Representative western blot showing IκBα and IκBβ in cytosolic fractions following exposure to LPS (30 m), or LPS after pretreatment (1 h) with BAY 11-7085 (5–20 μM) or parthenolide (5–20 μM) 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 μM) 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 μM) with lamin B as the loading control. (E) Densitometric evaluation (mean±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 μM). 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 IκBβ siRNA, western blotting and quantitative real-time PCR (qRT-PCR) confirmed a ∼50% reduction in IκBβ mRNA and protein expression (Fig. 4A,B). No significant effect on IκBα mRNA or protein expression was observed (Fig. 4A,B). Silencing IκBβ expression significantly reduced LPS-induced IL6, IL1β and IL12β expression (Fig. 4C; unadjusted data available in supplementary material Fig. S3). In contrast, silencing IκBβ had a variable effect on LPS-induced expression of other NFκB targets assessed (TNFα, MnSOD, IκBα, A20, RANTES, CXCL1 and IP-10; Fig. 4C; unadjusted data available in supplementary material Fig. S3). These results show that there are specific NFκB target genes whose LPS-induced expression requires first cytosolic IκBβ degradation, followed by subsequent nuclear IκBβ translocation.

IκBβ knock-in mouse embryonic fibroblasts have shortened LPS-induced NFκB activation

To further establish a relationship between IκBβ and the expression of specific LPS-induced NFκB-regulated target genes, we performed experiments using MEFs derived from IκBβ knock-in (AKBI) mice. In AKBI mice, IκBβ cDNA replaces the IκBα gene,

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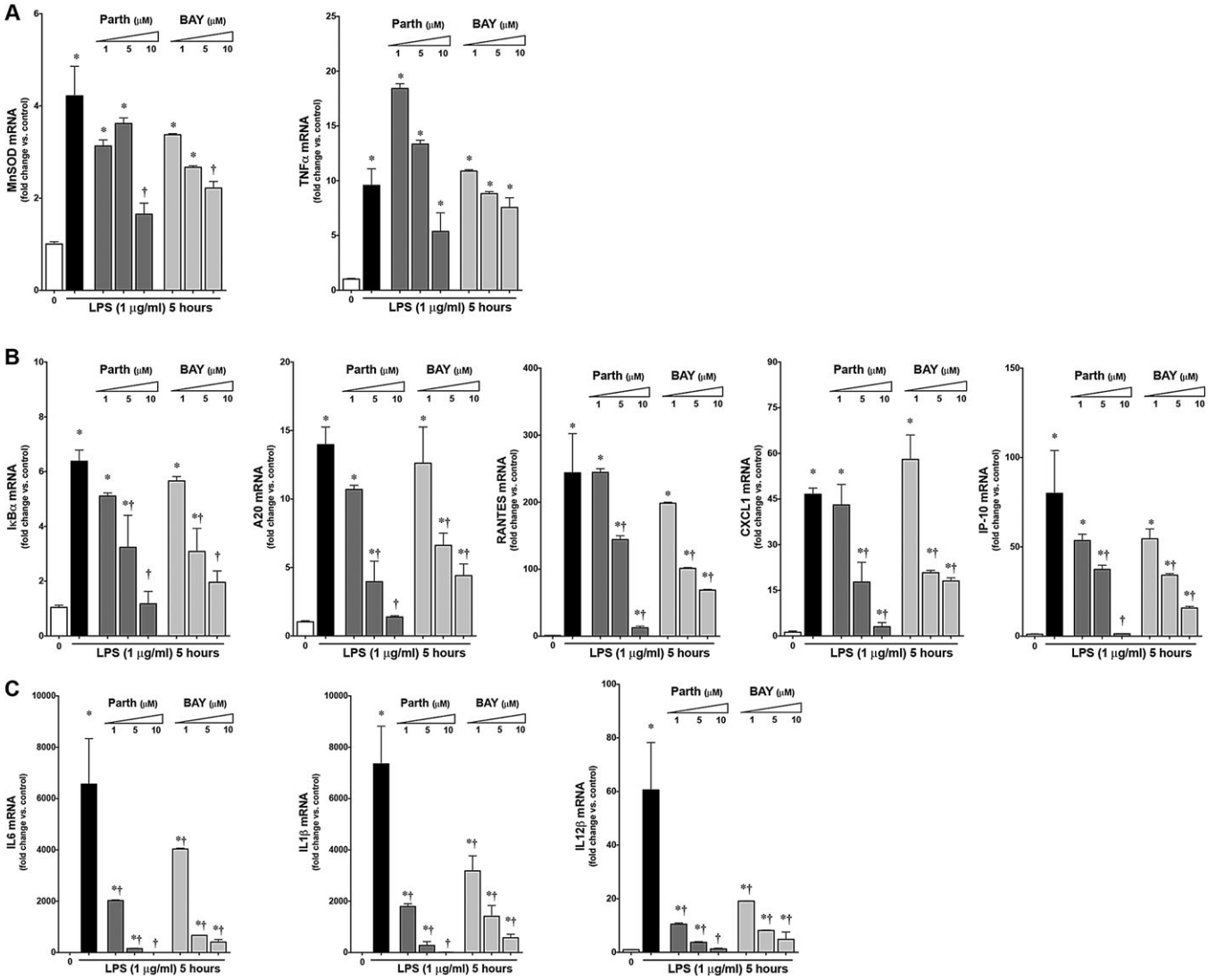

Fig. 2. Low-dose pharmacological NFκB inhibitors have differing effects on LPS-induced NFκB target genes in RAW 264.7 macrophages. Fold-increase of gene expression of (A) MnSOD and TNFα, (B) IκBα, A20, RANTES, CXCL1 and IP-10, and (C) IL1β, IL6 and IL12β following exposure to LPS (1 μg/ml, 5 h), or LPS after pretreatment (1 h) with parthenolide (1, 5 or 10 μM) or BAY 11-7085 (1, 5 or 10 μM). Values are mean±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 IκBβ and do not express IκBα (Cheng et al., 1998). Studies of these IκBβ knock-in, or AKBI, mice, showed that these mice were phenotypically indistinct from their wild-type controls. Furthermore, because the kinetics of TNFα-stimulated NFκB activity are similar between WT and AKBI MEFs (Cheng et al., 1998), it has been determined that IκBα and IκBβ show 'functional redundancy'. Importantly, neither LPS-induced NFκB activity nor gene expression was assessed in that study (Cheng et al., 1998). First, because altered expression of the IκB family of proteins can affect expression of other NFκB family member proteins, we assessed the expression of key NFκB signaling proteins. In AKBI MEFs, expression of IKKα, IKKβ, IKKε, p105 (large NFκB subunit encoded by *NFKB1*), p65, p50 (small NFκB 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 IκBα and overexpressed IκBβ compared to WT MEFs (supplementary material Fig. S4A,B). These results show that the expression of key NFκB signaling proteins is similar between WT and AKBI MEFs.

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

To assess the implications of rapid cytosolic re-accumulation of IκBβ following exposure to LPS, nuclear extracts were assessed for translocation of the NFκB 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).

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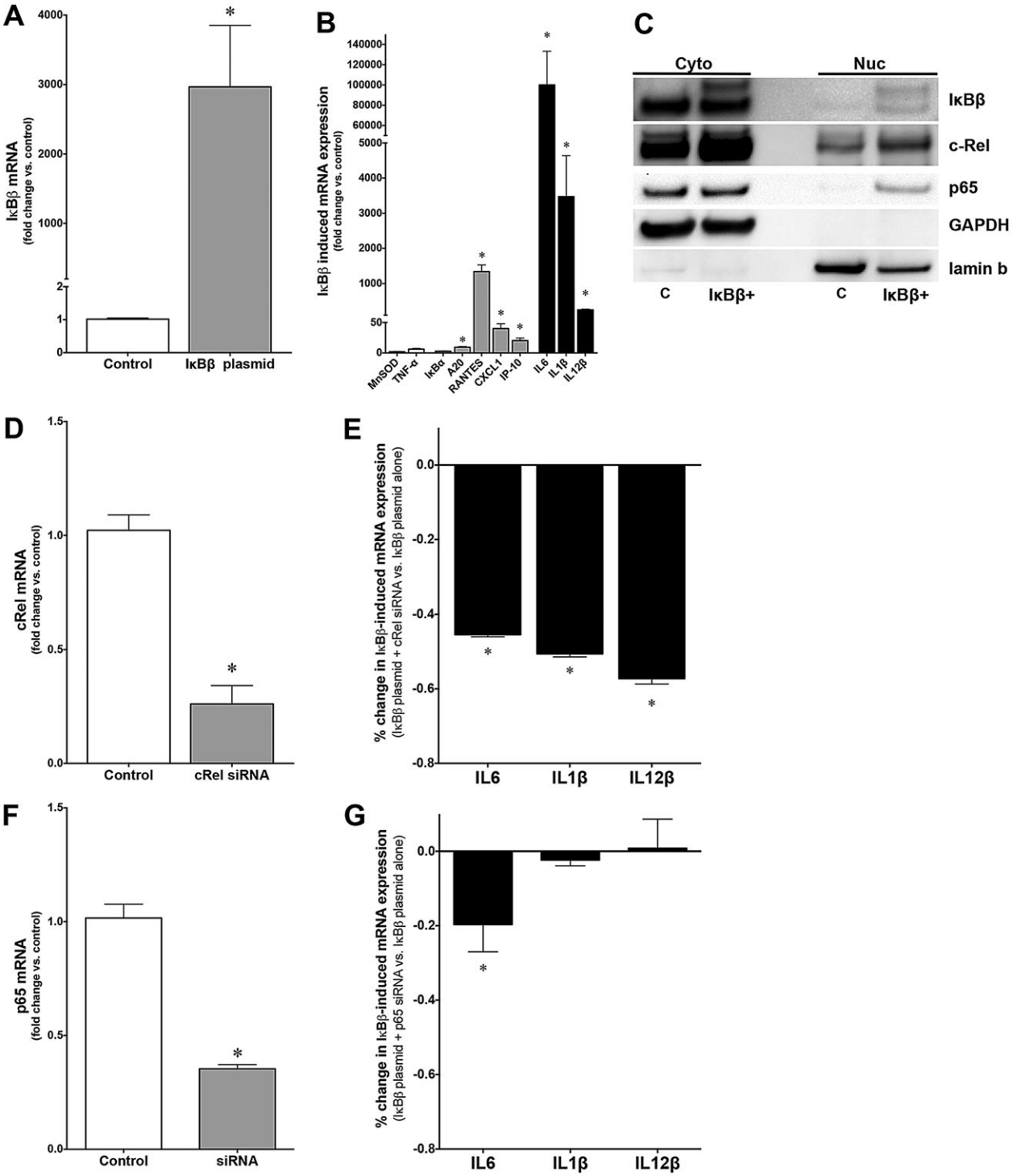

Fig. 3. IκBβ overexpression causes cRel dependent expression of NFκB target genes. (A) Relative IκBβ mRNA expression in RAW 264.7 macrophages following transfection with FLAG-tagged IκBβ expression plasmid. Values are means±s.e.m., *n*=9. **P*<0.05 versus untransfected control. (B) Relative mRNA expression of target genes in RAW 264.7 macrophages following transfection with FLAG-tagged IκBβ expression plasmid. Values are means+s.e.m., *n*=6. **P*<0.05 versus untransfected control. (C) Representative western blot showing IκBβ, cRel and p65 in cytosolic and nuclear extracts from RAW 267.4 macrophages transfected with IκBβ 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±s.e.m., *n*=6. **P*<0.05 versus untransfected control. (E) Percentage change in IκBβ-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 IκBβ expression plasmid with cRel siRNA transfected versus cells transfected with the IκBβ expression plasmid alone. Values are means±s.e.m., *n*=3 per time point. **P*<0.05 versus IκBβ expression plasmid alone. (F) Relative p65 mRNA expression in RAW 264.7 macrophages following transfection with p65 siRNA. Values are means±s.e.m., *n*=6. **P*<0.05 versus untransfected control. (G) Percentage change in IκBβ-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 IκBβ expression plasmid with cRel siRNA transfected versus cells transfected with the IκBβ expression plasmid alone. Values are means±s.e.m., *n*=3 per time point. **P*<0.05 versus IκBβ expression plasmid alone.

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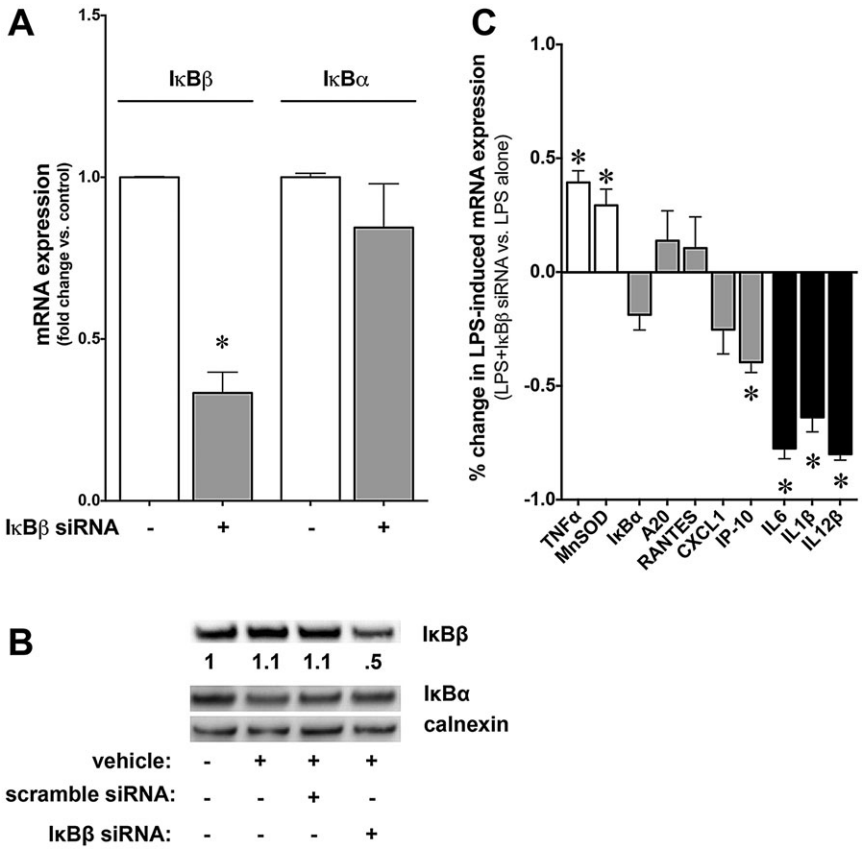

Fig. 4. IκBβ knockdown attenuates LPS-induced expression of select NFκB target genes. (A) Relative mRNA expression of IκBα and IκBβ in RAW 264.7 macrophages following transfection with IκBβ siRNA. Values are mean±s.e.m., n=9. *P<0.05 versus untransfected control. (B) Representative western blot showing IκBα and IκBβ in cytosolic extracts from RAW 267.4 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.

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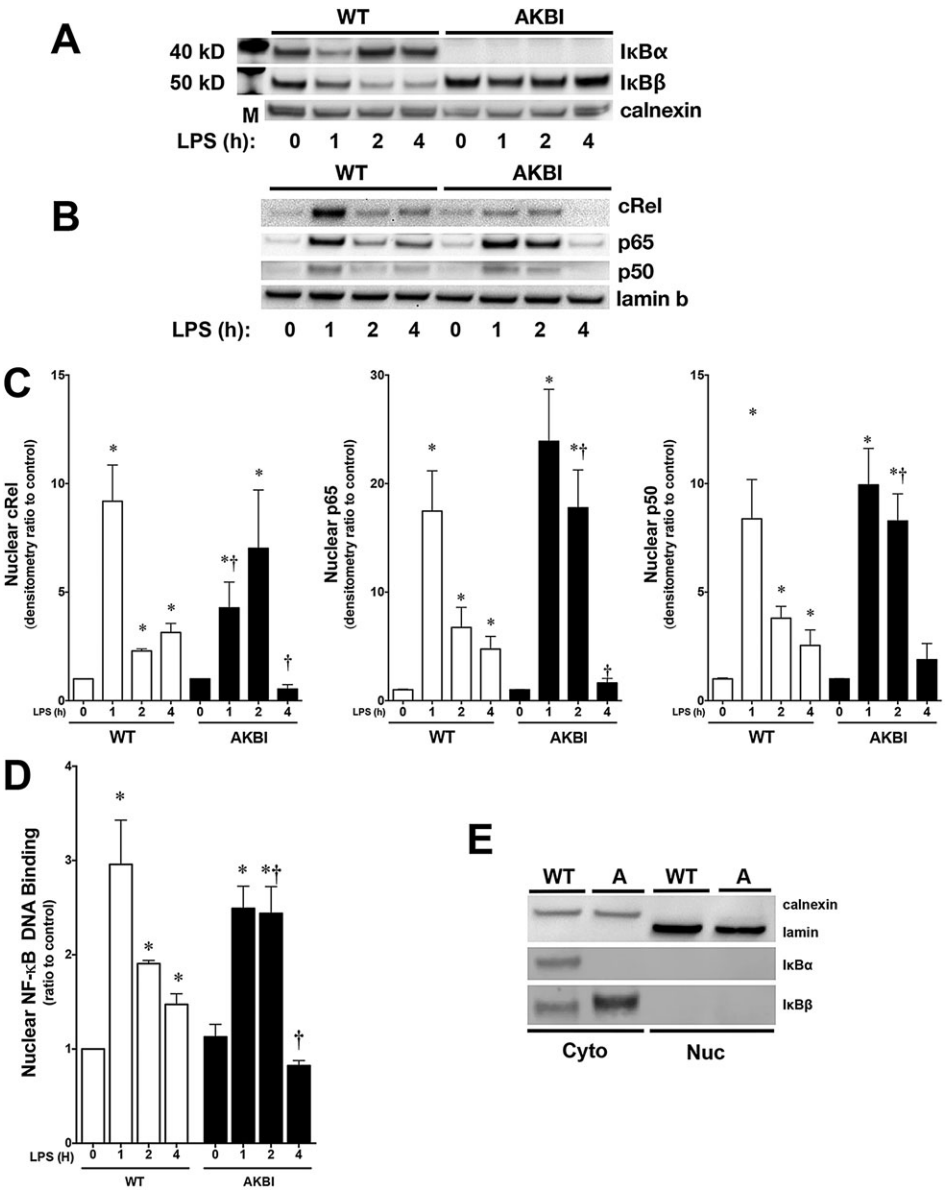

Fig. 5. AKBI MEFs demonstrate abbreviated LPS-induced NFκB activation.
(A) Representative western blot showing cytosolic IκBβ and IκBα in WT and AKBI MEFs exposed to LPS (1 µg/ml, 1–4 h). Calnexin is shown as a loading control. (B) Representative western blot showing nuclear cRel, p65 and p50 in WT and AKBI MEFs exposed to LPS (1 µg/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 µg/ml, 1–4 h). Results are expressed as a ratio to unexposed control and normalized to the lamin B loading control. Values are mean±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 NFκB DNA binding in WT and AKBI MEFs exposed to LPS as determined by a transcription factor assay. Values are mean±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 IκB 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 IκBβ 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 µM) attenuated LPS-induced IL6 expression in a dose-dependent manner (Fig. 8A). At a dose that significantly attenuated IL6 expression (100 µM, Fig. 8A), SN75 had no effect on LPS-induced TNFα or MnSOD expression (Fig. 8B). Furthermore, examination of nuclear extracts showed that SN75 (200 µM) 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 IκBβ nuclear translocation is sufficient to attenuate LPS-induced IL6 expression.

DISCUSSION

We found that both pharmacological and genetic manipulations of LPS-induced IκBβ–NFκB signaling affected the expression of a similar and unique subset of NFκB target genes in macrophages and MEF cells. Using low-dose parthenolide and BAY 11-7085, both well-known and widely used NFκB inhibitors, we show dose-dependent and differential effects on LPS-induced IκBα and IκBβ degradation. At low doses, pharmacological NFκB blockade inhibits only IκBβ degradation, and attenuates the expression of a specific subset of NFκB target genes including those encoding IL6, IL1β and IL12β. Using IκBβ siRNA and expression plasmids to perform gain- and loss-of-function experiments, we revealed a necessary and sufficient role played by IκBβ in the expression of these genes. Taken together, these studies demonstrate that both cytosolic degradation of IκBβ and the nuclear activity of IκBβ are important aspects of IκBβ–NFκB signaling. Both events – cytosolic degradation and subsequent nuclear translocation – contribute to the sustained expression of select NFκB target genes. Furthermore, using MEFs and BMDMs derived from WT and IκBβ

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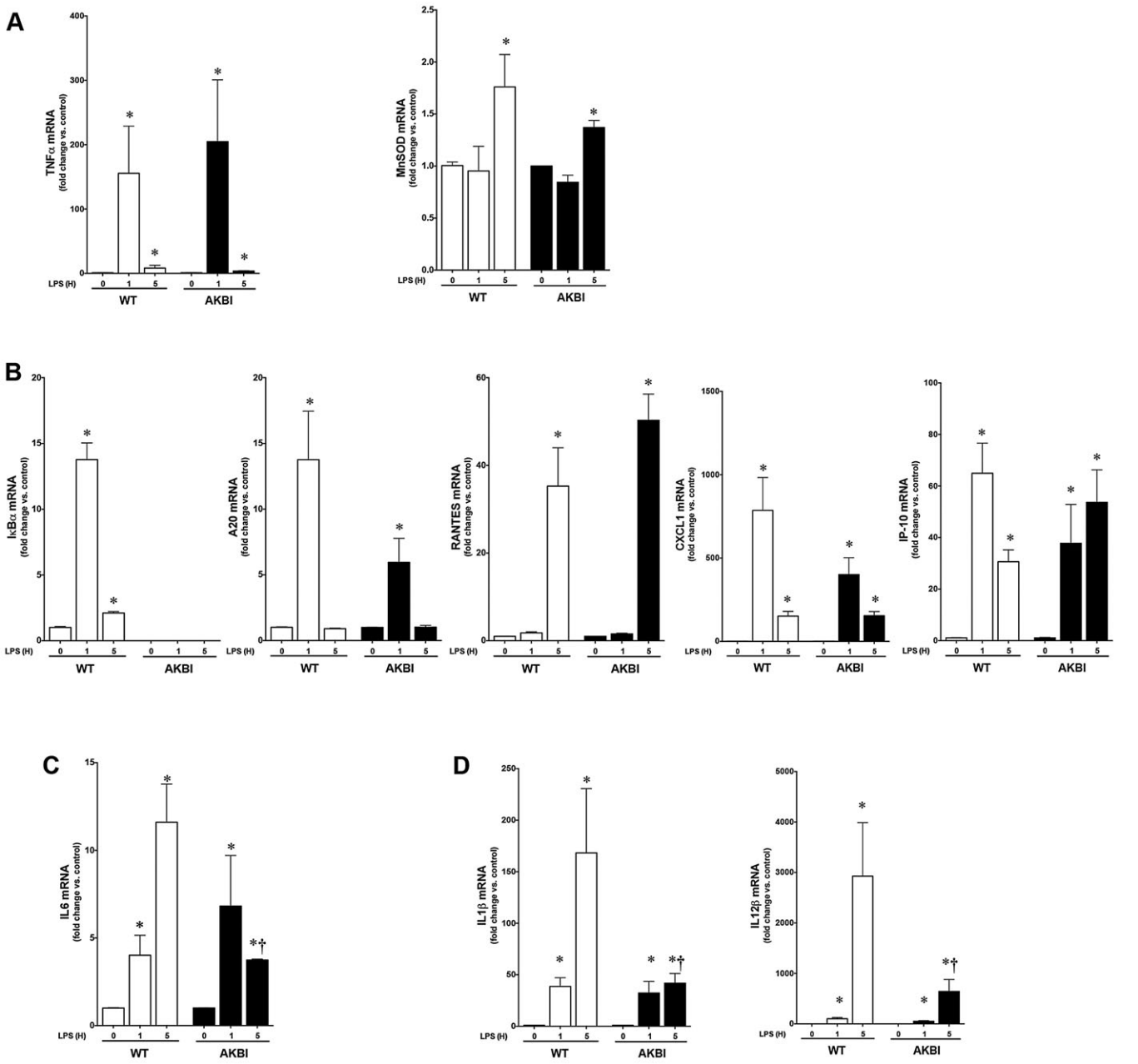

Fig. 6. AKBI MEFs demonstrate attenuated LPS-induced expression of select NFκB target genes. Fold increase of gene expression of (A) MnSOD and TNFα, (B) IκBα, A20, RANTES, CXCL1 and IP-10, and (C) IL6 following exposure to LPS (1 µg/ml, 1 and 5 h) in WT and AKBI MEFs. (D) Fold increase of gene expression of IL1β and IL12β following exposure to LPS (1 µg/ml, 1 and 5 h) in WT and AKBI BMDMs. Values are means±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 IκBβ following LPS abbreviates the duration of NFκB activation, and attenuates the expression of the same target genes that are sensitive to low-dose pharmacological IKK blockade. Further confirming the role of IκBβ and its preferred NFκB 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 IκBβ in mediating the expression of key NFκB target genes following LPS exposure. We prove that LPS-induced NFκB activation can be pharmacologicalally 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, IκBβ-mediated NFκB 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 NFκB 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; Ulloa et al., 2002). Despite this, no therapies that inhibit NFκB activity are

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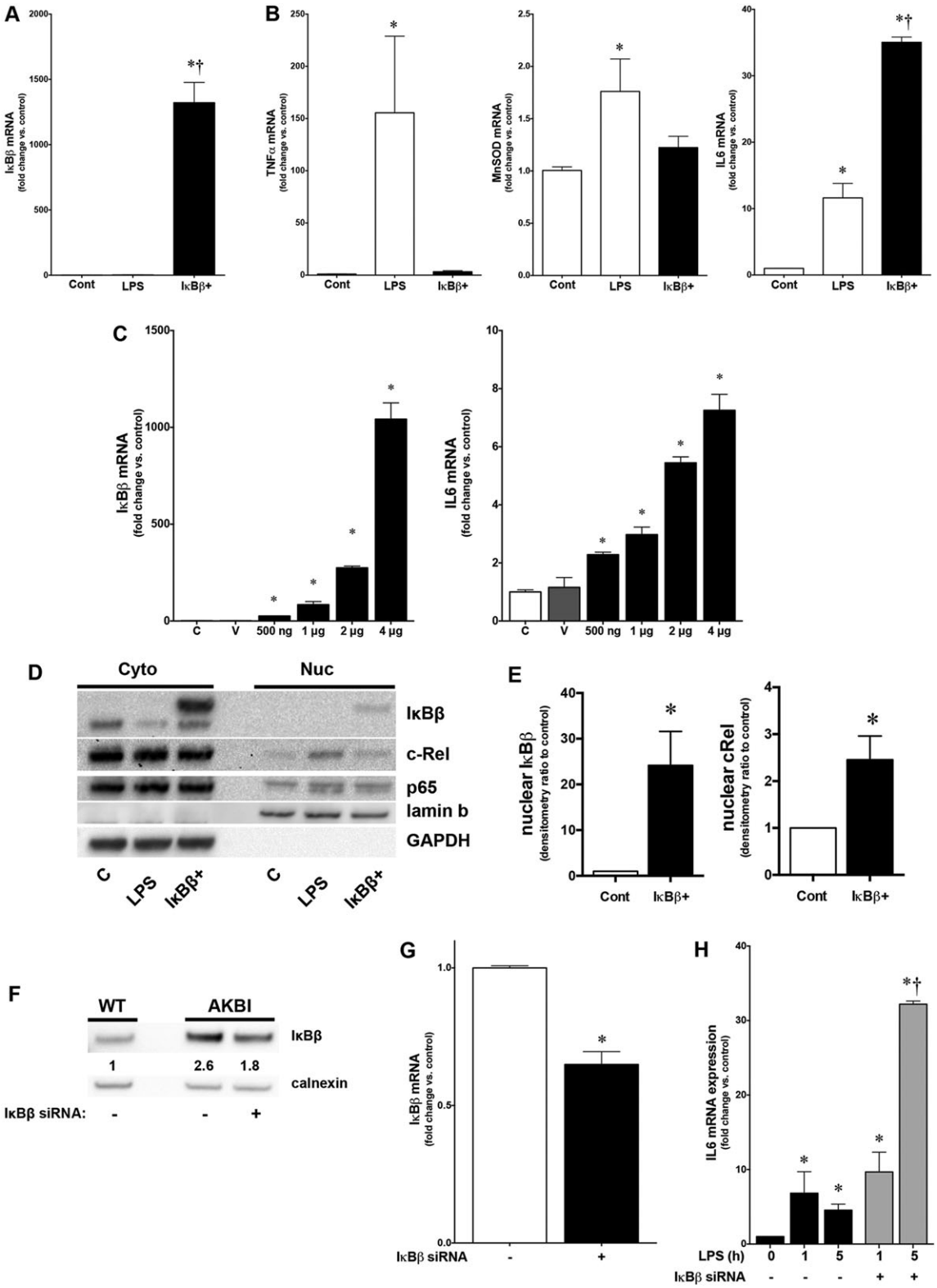

Fig. 7. See next page for legend.

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

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

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

Interestingly, we could find no reports evaluating the effect of pharmacological inhibitors of NFκB activity on IκBβ–NFκB signaling following LPS-exposure or endotoxemic shock. Recently, two separate laboratories have demonstrated that IκBβ$^{-/-}$ mice are extremely resistant to endotoxemic shock, a finding explained by attenuated expression of key pro-inflammatory NFκB target genes including IL6, IL1β and IL12β (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$^{-/-}$ mice show increased sensitivity to polymicrobial sepsis (Courtine et al., 2011). However, cRel$^{-/-}$ 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 IκBβ and cRel inform our understanding of the subtleties underlying the NFκB response to inflammatory stress.

Multiple pharmacological inhibitors of NFκB 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 NFκB inhibitor demonstrated that a dose of 20 µM was sufficient to inhibit IκBα degradation (Pierce et al., 1997). Importantly, the effect on IκBβ degradation was not assessed. Since this report, these agents have become so widely accepted as NFκB inhibitors that their effect on IκBα degradation is frequently not reported. Scattered data suggests that there is a dose-dependent effect of these inhibitors on NFκB signaling. Parthenolide pretreatment at doses >10 µM inhibits LPS-induced IκBα degradation (Yip et al., 2004), p65 nuclear translocation (Li et al., 2006) and NFκB reporter activity in RAW 264.7 cells (Park et al., 2011). Interestingly, parthenolide has been reported to attenuate LPS-induced expression of NFκB target genes in a dose-dependent manner (at doses of 0.4–4 µM) lower than those previously demonstrated to inhibit IκBα 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 IκBβ 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, IκBβ-mediated NFκB activation and gene expression can be pharmacologically targeted.

The observation that low-dose pharmacological inhibition of IKK specifically attenuates IκBβ degradation could be explained by the affinity of the IKK complex for IκBα and IκBβ. Previous reports indicate that the kinetics of IκBβ degradation is unique and slower than that of IκBα following LPS stimulation (Thompson et al., 1995). Additionally, IKK is known to have a lower affinity for IκBβ when compared to IκBα (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 IκBβ. 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 NFκB target genes. However, we chose a diverse set of LPS-induced, pro-inflammatory NFκB targets that were either MyD88 independent (IP10, RANTES) or MyD88 dependent (IL1β, CXCL1, TNF, IL12β, IL6). (Björkbacka et al., 2004). Additionally, NFκB target genes that regulate NFκB activity (IκBα, A20) and antioxidant enzymes (MnSOD) were assessed (Gilmore and Herscovitch, 2006; Verstrepen et al., 2010). Undoubtedly, LPS-induced expression of other NFκB 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, IκBβ-mediated NFκB activation.

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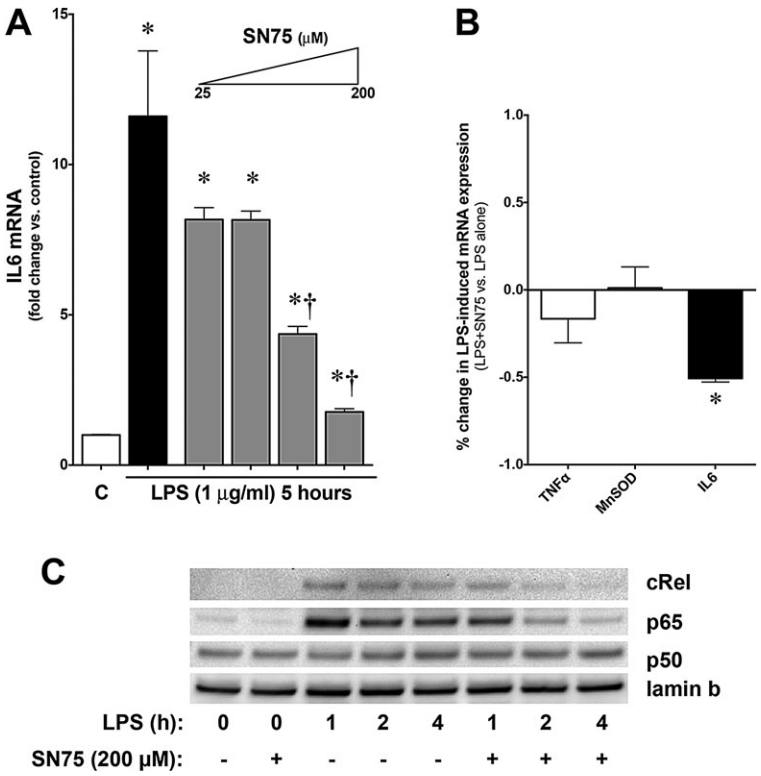

Fig. 8. Inhibition of LPS-induced cRel nuclear translocation attenuates IL6 expression. (A) Relative IL6 mRNA expression in WT MEFs following exposure to LPS (1 μg/ml, 5h) or following pre-treatment with a cell-permeable synthetic peptide containing the cRel nuclear localization sequence (SN75, 25–200 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-IκBa vector

(Clontech) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To achieve IκBβ, cRel and p65 silencing, cells were transfected with siRNA (Dharmacon catalog numbers: IκBβ 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 predesigned, species-appropriate exon-spanning primers (catalog numbers are as follows: MnSOD, Mm00449726_m1; TNF, Mm00443258_m1; IκBα, Mm00477798_m1; A20, Mm00437121_m1; RANTES, Mm01302428_m1; CXCL1, Mm04207460_m1; IP10, Mm00445235_m1; IL1β, Mm01336189_m1; IL6, Mm00446190_m1; IL12β, 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 (ΔΔCt) 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 IκKα (Cell Signaling 2682), IKKβ (Cell Signaling 2370), IKKε (Cell Signaling 3416), IκBα (Santa Cruz Biotechnology 371), IκBβ (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 NFκB binding by a transcription factor assay

A NFκB-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 NFκB 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, QRKRKLMP) 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 μg/ml or 200 μg/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
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Supplemental Figure Legends.

Supplemental Figure 1: IκBα over-expression does not induce expression of cRel dependent expression of NFκB target genes

(A) Relative IκBα, IL6, IL1β and IL12 β mRNA expression in RAW 264.7 macrophages following transfection with IκBα expression plasmid. Values are means ± SEM. n=9. (B) Relative LPS-induced (1 μg/ml, 5 hrs), IL6, IL1β, and IL12β mRNA expression in RAW 264.7 macrophages (cont) and following transfection with IκBβ expression plasmid (IκBβ+). Values are means ± SEM. n=3. *, $p<0.05$ vs. untransfected LPS exposed.

Supplemental Figure 2: IκBβ over-expression induces expression of cRel but not p65

(A) Relative cRel and p65 mRNA expression in RAW 264.7 macrophages following transfection with IκBβ expression plasmid. Values are means ± SEM. n=9. *, $p<0.05$ vs. untransfected controls. (B) Representative Western blot showing whole cell lysate levels of IκBβ, c-Rel and p65 proteins in control RAW 264.7 (C) and in RAW 264.7 transfected with IκBβ expression plasmid (IκBβ+). Calnexin shown as loading control. Densitometric evaluation of protein expression normalized to control is provided.

Supplemental Figure 3: Unadjusted data presented in Figure 3E and 4C.

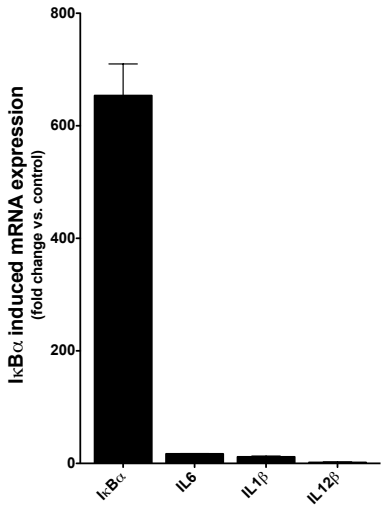
(A) Data presented in Figure 3E: Relative mRNA expression of target genes in RAW 264.7 macrophages following transfection with FLAG-tagged IκBβ expression plasmid (IκBβ+) or FLAG-tagged IκBβ expression plasmid and cRel siRNA (IκBβ+cRel-). Values are means+SEM. n=3. *, $p<0.05$ vs. untransfected control; †, $p<0.05$ vs. IκBβ expression plasmid alone. (B) Data presented in Figure 4C: Fold-increase of gene expression of MnSOD, TNFα, IκBα, A20, RANTES, CXCL1, IP-10, IL1β, IL6 and IL12β following exposure to LPS (1 μg/ml, 5 hrs), or LPS after transfection with IκBβ siRNA. Values are means ± SEM. n=3/time point. *, $p<0.05$ vs. unexposed control; †, $p<0.05$ vs. LPS-exposed.

Supplemental Figure 4: Expression of NFκB signaling pathway proteins is not different between AKBI and WT MEF

(A) Representative Western blot showing WT and AKBI lysate control levels of IKKα, IKKβ, IKKε, IκBβ, IκBα, p65, p50, and c-Rel proteins with calnexin as loading control. Densitometric evaluation of protein expression normalized to WT is provided. (B) Relative IKKα, IKKβ, IKKε, IκBβ, IκBα, p50, c-Rel, TLR4 and MyD88 mRNA levels. Values are means ± SEM. n=8. *, $p<0.05$ vs. WT.

Supplemental Figure 1

A.



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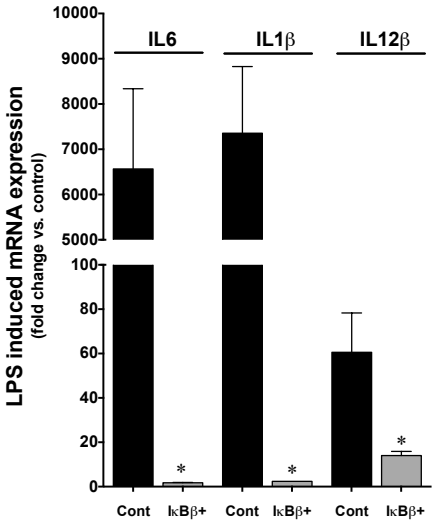
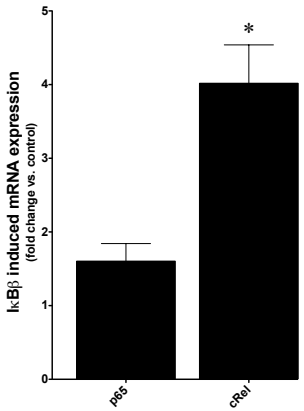


Supplemental Figure 2

A.



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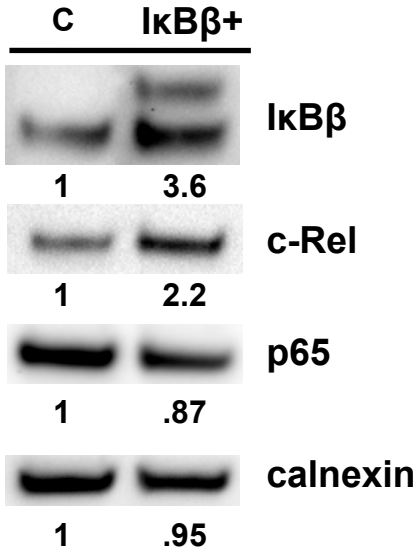
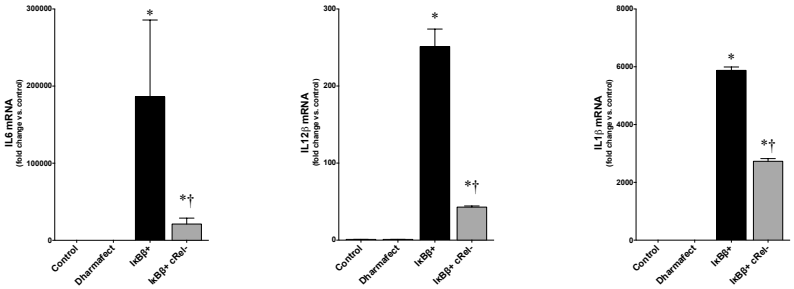


Supplemental Figure 3

A.



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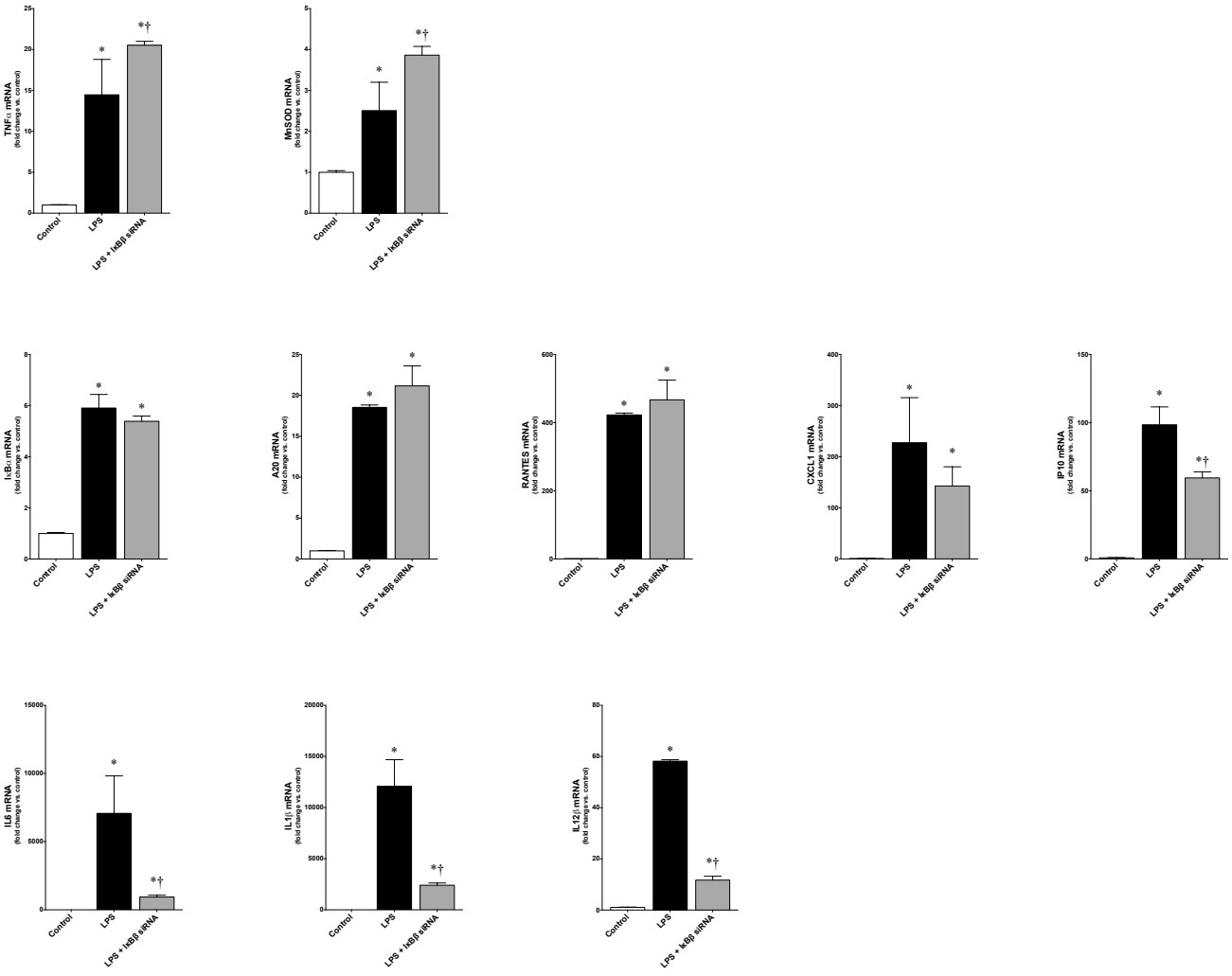
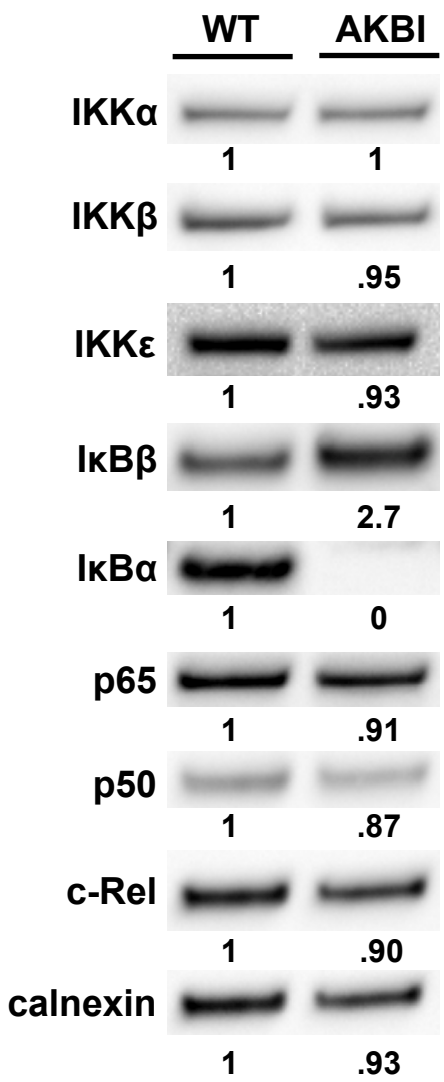


Supplemental Figure 4

A.



B.