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Inhibition of IκBβ/NFκB signaling prevents LPS-induced IL1β expression without increasing apoptosis in the developing mouse lung

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BACKGROUND: The pro-inflammatory consequences of IL1β expression contribute to the pathogenesis of bronchopulmonary dysplasia. Selectively targeting Lipopolysaccharide (LPS)-induced IκBβ/NFκB signaling attenuates IL1β mRNA expression in macrophages. Whether targeting IκBβ/NFκB signaling affects the anti-apoptotic gene expression, a known consequence of global LPS-induced NFκB inhibition, is unknown.

METHODS: Macrophages (RAW 264.7, bone marrow-derived macrophage) were assessed for LPS-induced IL1β mRNA/protein expression, anti-apoptotic gene expression, cell viability (trypan blue exclusion), and activation of apoptosis (caspase-3 and PARP cleavage) following pharmacologic and genetic attenuation of IκBβ/NFκB signaling. Expressions of IL1β and anti-apoptotic genes were assessed in endotoxemic newborn mice (P0) with intact (WT), absent (IκBβ KO), and attenuated (IκBβ overexpressing) IκBβ/NFκB signaling.

RESULTS: In cultured macrophages, pharmacologic and genetic inhibition of LPS-induced IκBβ/NFκB signaling significantly attenuated IL1β mRNA and protein expression. Importantly, targeting IκBβ/NFκB signaling did not attenuate LPS-induced expression of anti-apoptotic genes or result in cell death. In endotoxemic neonatal mice, targeting LPS-induced IκBβ/NFκB signaling significantly attenuated pulmonary IL1β expression without affecting the anti-apoptotic gene expression.

CONCLUSION: Targeting IκBβ/NFκB signaling prevents LPS-induced IL1β expression without inducing apoptosis in cultured macrophages and in the lungs of endotoxemic newborn mice. Inhibiting this pathway may prevent inflammatory injury without affecting the protective role of NFκB activity in the developing lung.

Bronchopulmonary dysplasia (BPD) is a leading cause of mortality in extremely preterm infants that survive past 1 month of age, and is an independent risk factor for long-term neurodevelopmental impairment (1). Evidence of an active, pro-inflammatory, innate immune response can be measured in the amniotic fluid, serum, and tracheal aspirates of infants that go on to develop BPD (2,3). Similar to the observations made in humans, inflammation is a central finding in every animal model of BPD (4–7). These clinical and laboratory findings support the hypothesis that inflammation is central to the pathogenesis of BPD.

Despite these associations, no safe and effective anti-inflammatory therapies are currently available to prevent BPD in at-risk infants. Understanding the mechanisms that link the innate immune response to neonatal lung injury is key to developing targeted anti-inflammatory therapies that minimize detrimental off-target effects. Multiple clinical and pre-clinical studies implicate the pro-inflammatory cytokine IL1β in the pathogenesis of BPD (2,3,8–12). To date, interventions to attenuate the pro-inflammatory effects of IL1β have targeted events downstream of increased gene expression. These include IL1 receptor antagonists, decoy IL1 receptors, and neutralizing IL1 antibodies (13). Pre-clinical studies demonstrate that IL1 receptor antagonists attenuate hyperoxia-induced lung injury in neonatal mice and rats (14–16). Although promising, these therapies may have unintended consequences in the developing lung. With IL1 receptor blockade, the effects of both IL1α and IL1β are inhibited. Importantly, IL1α is constitutively expressed in the developing lung, and is known to directly stimulate surfactant synthesis (17). Thus, inhibiting the activity of constitutively expressed IL1α through IL1 receptor blockade may be uniquely detrimental in the neonatal period.

Inflammasome-mediated post-translational processing is the penultimate step in IL1β activity; however, NFκB-mediated transcription is the rate-limiting step of IL1β release (13). Thus, targeting NFκB activity could attenuate stimulus-induced IL1β production and inflammatory-stress-induced neonatal lung injury. However, NFκB activity is responsible for the expression of multiple proteins that are responsible for normal growth and development, as well as anti-apoptotic factors that prevent cell death following exposure to LPS (18,19). This finding likely explains the lack of an apoptotic response in the developing lung of sheep, rabbits,

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and mice exposed to LPS (20–22). Therefore, it is not surprising that pre-clinical studies show that complete NFκB inhibition exacerbates neonatal lung injury induced by endotoxemia (20,23). It remains unknown whether NFκB activity can be manipulated to attenuate the pro-inflammatory response and IL1β expression, while leaving protective anti-apoptotic signals intact.

Unique characteristics of the NFκB inhibitory proteins, IκBα and IκBβ, mediate inflammatory stress-induced IL1β expression. In quiescent cells, the NFκB inhibitory proteins IκBα and IκBβ sequester NFκB in the cytoplasm. Following exposure to LPS, these proteins are phosphorylated by IKK and are degraded, allowing NFκB nuclear translocation. Following degradation, 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 (24). In contrast, IκBβ lacks a nuclear export sequence, and remains in the nucleus to facilitate NFκB DNA binding (25). Thus, the unique nuclear activity of IκBβ dictates sustained expression of select target genes, including IL1β (25,26).

Recently, we have demonstrated that IκBβ/NFκB signaling can be pharmacologically targeted *in vitro*, while leaving IκBα/NFκB signaling intact (27–30). By preventing the nuclear activity of IκBβ, the sustained expression of key pro-inflammatory target genes (COX-2, ET-1, IL1β) is significantly reduced (27–30). In contrast, by allowing IκBα/NFκB signaling to proceed, the expression of key antioxidant (MnSOD) and anti-inflammatory (A20) target genes that are not dependent upon nuclear IκBβ activity is unimpaired (28). We undertook this study to determine whether IκBβ/NFκB signaling could be targeted to prevent the expression of IL1β without increasing apoptosis in macrophages and the newborn lung.

METHODS

Murine Model of Endotoxemia

Newborn (P0) ICR (WT), and IκBβ-overexpressing (AKBI) mice were exposed to 0–50 mg/kg LPS (IP) and their survival was monitored to identify lethal and sublethal endotoxemia. Newborn ICR (WT; background strain of AKBI) and IκBβ-overexpressing (AKBI) mice and C57B6 (WT; background strain of KO) and IκBβ-knockout (KO) mice were exposed to sub-lethal endotoxemia (LPS (Sigma-Aldrich, St. Louis, MO, L2630); 5 mg/kg IP). Both AKBI and $I\kappa B\beta^{-/-}$ had normal litter sizes and were phenotypically indistinct from their WT controls. (25,31) The mice were killed and normal saline was perfused through the right ventricle, and liver and lung samples were collected and processed as described below. All procedures were approved by the IACUC at the University of Colorado (Aurora, CO).

Cell Culture, LPS Exposure, and Pharmacologic NFκB Inhibition

RAW 264.7 murine macrophages (ATCC, Manassas, VA) were cultured according to the manufacturer's instructions. Cells were exposed to LPS (1 μg/ml, Sigma L6529). To pharmacologically inhibit NFκB activation, the cells were exposed to Bay 11-7085 (0.5–20 μM, Sigma) for 1 h prior to LPS exposure.

WT (ICR and C57B6), AKBI, and KO bone marrow-derived macrophages (BMDMs) were cultured as previously described (32) and exposed to LPS (1 μg/ml) for 1–24 h.

Evaluation of Cell Death Using Trypan Blue Exclusion

Trypan blue exclusion was used to determine cell viability as previously described (33).

Whole-cell lysate from cultured cells

Whole-cell lysates were collected from cultured cells, and protein concentration was determined as previously described (33).

Immunoblot Analysis

Lysates were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen, Carlsbad, CA) and proteins were transferred to an Immobilon membrane (Millipore, Billerica, MA). The membranes were blotted with anti-IL1β (Cell Signaling Technology, Danvers, MA, #12426), anti-IκBβ (Santa Cruz Biotechnologies, Dallas, TX, #9130), anti-PARP (Cell Signaling Technology #9542), anti-Caspase-3 (Cell Signaling Technology #9665), and anti-Calnexin (Enzo Life Sciences, Farmingdale, NY, ADI-SPA-860). Densitometric analysis was performed using Image Studio (LI-COR, Lincoln, NE).

Analysis of Relative mRNA Levels by RT-qPCR

Pulmonary mRNA was collected using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. RNA was assessed for purity and concentration using the NanoDrop (ThermoFisher Scientific, Waltham, MA), and cDNA was synthesized using the Verso cDNA synthesis Kit (Thermo Scientific). Relative mRNA levels were evaluated by quantitative real-time PCR using the TaqMan gene expression system (Applied Biosystems, Foster City, CA). Gene expressions of BCL2A1, BCL-XL, XIAP, BIRC3, PAI2, and IL1β genes were assessed with predesigned exon-spanning primers (Mm03646861_mH, Mm00437783_m1, Mm01248390_m1, Mm01168413_m1, Mm00440905_m1, and Mm01336189_m1) using the StepOnePlus Real Time PCR System (Applied Biosystems). Relative quantitation was performed through normalization to the endogenous control 18S using the cycle threshold (ΔΔCt) method.

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, LPS exposure), with statistical significance between and within groups determined by means of Bonferroni method of multiple comparisons (InStat, GraphPad Software, La Jolla, CA). Statistical significance was defined as $P < 0.05$.

RESULTS

Inhibiting IκBβ/NFκB Signaling Attenuates LPS-Induced IL1β mRNA and Protein Expression

We have previously demonstrated that low doses of the IKK inhibitor BAY 11-7085 (1 μM) selectively targets IκBβ/NFκB signaling and attenuates the expression of select pro-inflammatory target genes (27–30). Extending this dose–response experiment, we determined that a minimum of BAY 11-7085 (0.5 μM) is necessary to significantly attenuate LPS-induced IL1β mRNA expression in RAW 264.7 cells (**Figure 1a**). Previous studies have demonstrated that RAW 264.7 cells do not secrete active IL1β due to absent expression of the inflammasome component ASC (apoptosis-associated speck-like protein containing a C-terminal caspase-activating recruiting domain) (34). Despite this, LPS-exposed RAW 264.7 cells actively produced pro-IL1β starting at 2 h of exposure (**Figure 1b**). Consistent with the inhibition of LPS-induced IL1β mRNA expression, we found that pretreatment

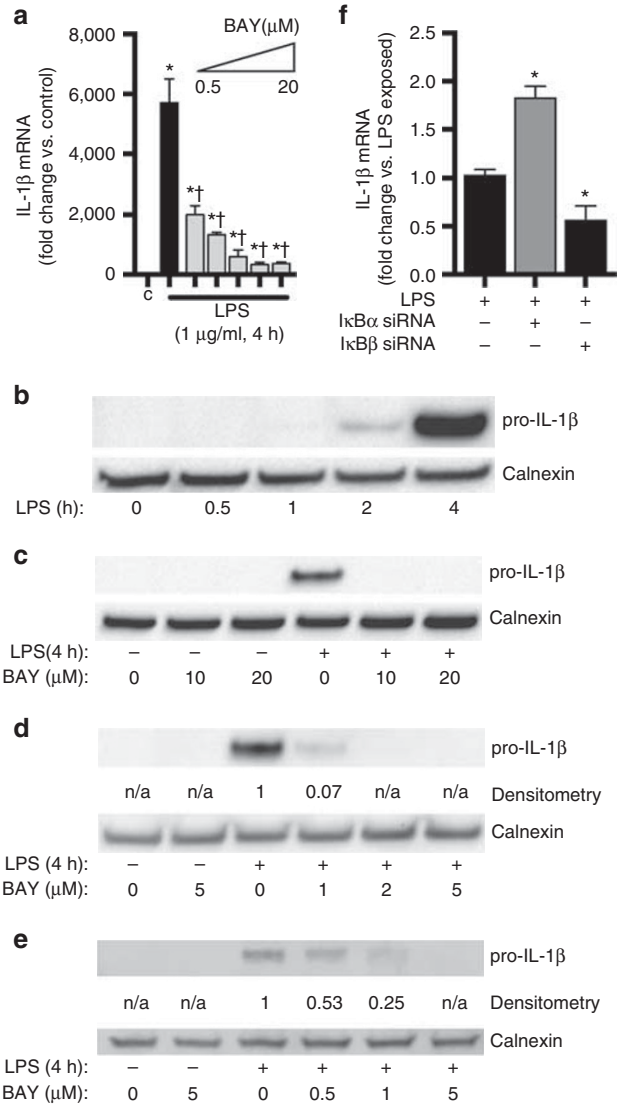

Figure 1. Inhibition of LPS-induced IκBβ/NFκB signaling attenuates IL1β expression in RAW 264.7 macrophages. (**a**) Fold-increase in IL1β expression following LPS exposure (1 μg/ml, 4 h) or pretreatment with BAY 11-7085 (0.5–20 μM, 1 h) and LPS exposure (1 μg/ml, 4 h). Values are means+SEM ($n = 4$–6/time point); h, hours; *$P < 0.05$ vs. unexposed control; †$P < 0.05$ vs. LPS exposed. Representative western blots showing (**b**) pro-IL1β protein expression following LPS exposure (1 μg/ml, 0.5–4 h), (**c**) pro-IL1β expression following LPS exposure (1 μg/ml, 4 h) or pretreatment with high-dose BAY-11-7085 (10–20 μM, 1 h) and LPS exposure (1 μg/ml, 4 h), and (**d**,**e**) pro-IL1β expression following LPS exposure or pretreatment with low-dose BAY 11-7085 (0.5–5 μM, 1 h) and LPS exposure (1 μg/ml, 4 h). Calnexin shown as loading control. Densitometry ratio to control is provided. (**f**) Percent-change in LPS-induced (1 μg/ml, 4 h) IL1β expression following transfection with either IκBα or IκBβ siRNA. Values are expressed as a ratio to LPS-induced levels and are means+SEM ($n = 4$–6/time point); *$P < 0.05$ vs. LPS exposed.

with high-dose BAY 11-7085 (10–20 μM) completely inhibited LPS-induced pro-IL1β protein expression (Figure 1c). In addition, significant dose-dependent inhibition of LPS-induced pro-IL1β expression could be achieved with low-dose BAY 11-7085 (0.5–1 μM) (Figure 1d,e). As pharmacologic agents may have unanticipated off-target effects, we

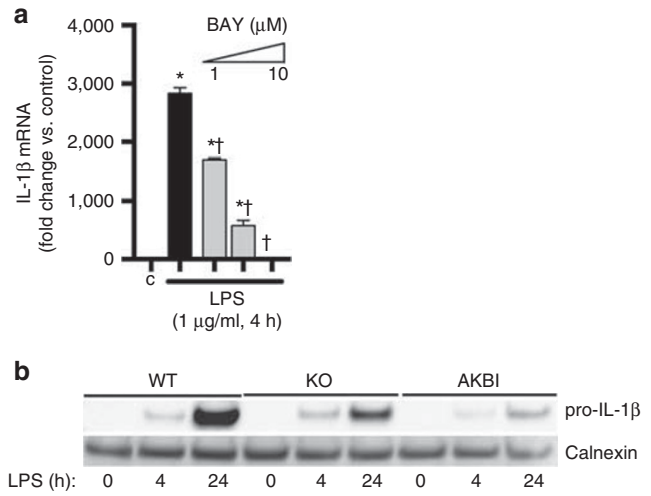

Figure 2. Both abbreviated and absent LPS-induced IκBβ/NFκB signaling attenuates IL1β expression in BMDM. (**a**) Fold-increase in IL1β expression following LPS exposure (1 μg/ml, 4 h) or pretreatment with BAY 11-7085 (1–10 μM) and LPS exposure (1 μg/ml, 4 h). Values are means+SEM ($n = 6$/time point); h, hours; *$P < 0.05$ vs. unexposed control; †, $P < 0.05$ vs. LPS exposed. (**b**) Representative western blots showing pro-IL1β expression following LPS exposure (1 μg/ml, 0–24 h) in WT, IκBβ-overexpressing (AKBI), or IκBβ-knockout (KO) BMDM. BMDM, bone marrow-derived macrophage.

assessed LPS-induced IL1β expression following transfection with siRNA targeting either IκBα or IκBβ. Through this approach, we reasoned that we could dissect the nuclear roles of IκBα or IκBβ in mediating IL1β expression. Specifically, we hypothesized that silencing IκBα expression would lead to ineffective termination of NFκB activity, thus increasing LPS-induced IL1β expression (Figure 1f). In contrast, silencing IκBβ expression would attenuate sustained NFκB activity, thus decreasing the LPS-induced IL1β expression (Figure 1f). Macrophages transfected with IκBα siRNA demonstrated a 70% increase in LPS-induced IL1β expression when compared with controls. In contrast, macrophages transfected with IκBβ siRNA demonstrated a 50% decrease in LPS-induced IL1β expression when compared with controls. Thus, targeting IκBβ/NFκB signaling attenuates LPS-induced IL1β mRNA and protein expression.

LPS-Induced IL1β Expression is Attenuated in AKBI and IκBβ$^{-/-}$ Macrophages

As RAW 264.7 murine macrophages are immortalized and may demonstrate unexpected aberrations in NFκB signaling, we sought to demonstrate a role of IκBβ/NFκB signaling in LPS-induced IL1β expression in BMDM. First, we confirmed that similar to RAW 264.7 cells, low-dose BAY 11-7085 significantly inhibits LPS-induced pro-IL1β expression in WT BMDM (Figure 2a). To further validate a mechanistic link between IκBβ/NFκB signaling and IL1β expression following exposure to LPS, we used BMDM isolated from mice genetically modified to have either abbreviated or absent IκBβ/NFκB signaling. Due to overexpression of the NFκB inhibitory protein IκBβ, AKBI BMDM demonstrated

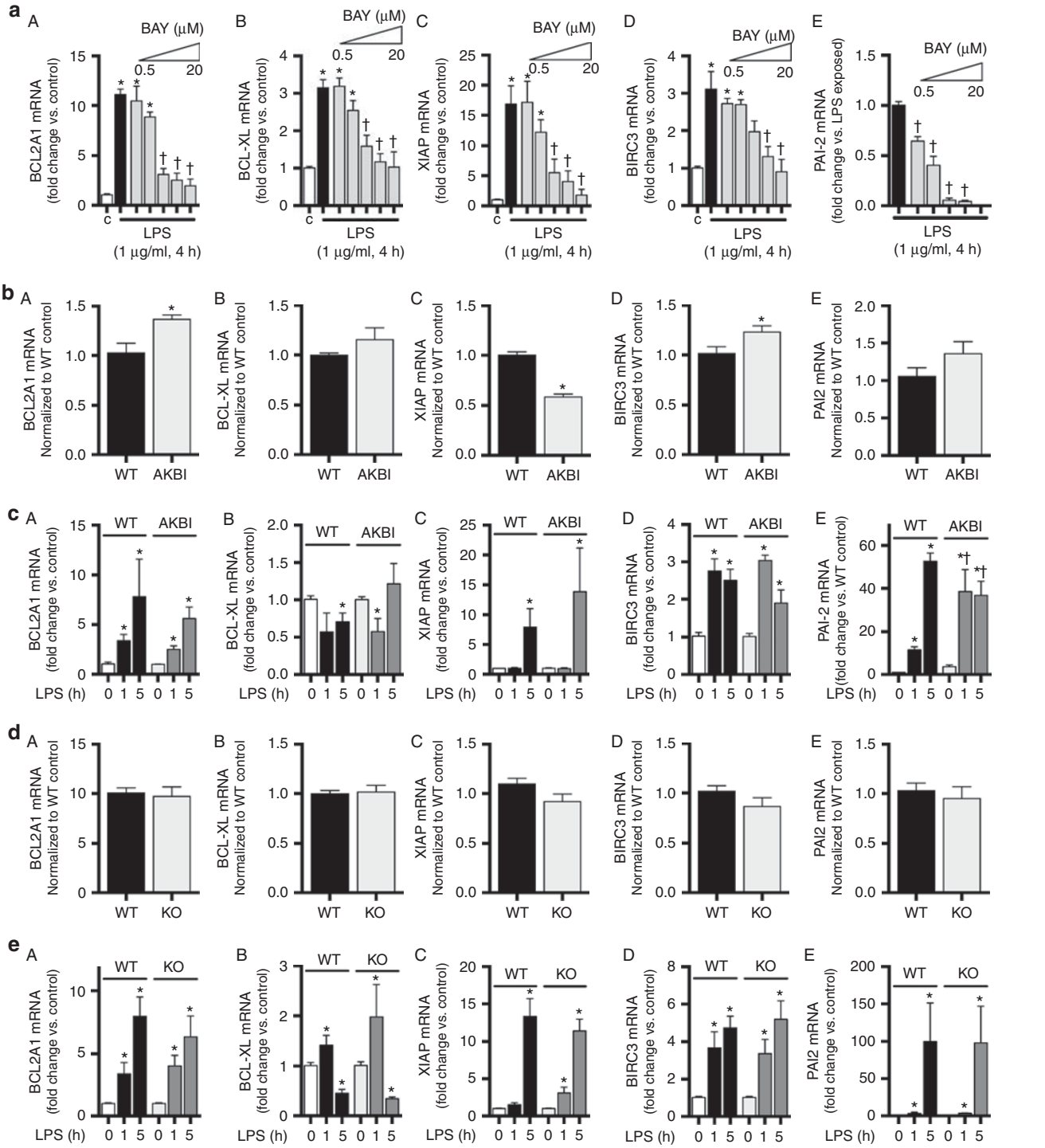

Figure 3. Targeting IκBβ/NFκB signaling does not attenuate LPS-induced expression of anti-apoptotic genes. Expression of (**A**) BCL2A1, (**B**) BCL-XL, (**C**) XIAP, (**D**) BIRC3, and (**E**) PAI2 in (**a**) RAW 264.7 macrophages following LPS exposure (1 μg/ml, 4 h) or pretreatment with BAY 11-7085 (0.5–20 μM, 1 h) and LPS exposure (1 μg/ml, 4 h). Values are means+SEM ($n = 6$/time point); h, hours; *, $P < 0.05$ vs. unexposed control; †, $P < 0.05$ vs. LPS exposed (**b**,**c**) WT or AKBI BMDM at (**b**) baseline or (**c**) following LPS exposure (1 μg/ml, 4 h). Values are means+SEM ($n = 6$/time point); h, hours; *, $P < 0.05$ vs. unexposed control; †, $P < 0.05$ vs. time-matched WT LPS exposed (**d**,**e**) WT or KO BMDM at (**d**) baseline or (**e**) following LPS exposure (1 μg/ml, 4 h). Values are means+SEM ($n = 6$/time point); h, hours; *, $P < 0.05$ vs. unexposed control; †, $P < 0.05$ vs. time-matched WT LPS exposed.

attenuated LPS-induced IκBβ/NFκB signaling (28). We have previously demonstrated that LPS-induced IL1β mRNA expression is attenuated at early time points (1–5 h), but protein expression was not evaluated (28). Due to the absence of IκBβ, IκBβ$^{-/-}$ BMDM are devoid of IκBβ/NFκB signaling (25). It has previously been observed that LPS-induced IL1β mRNA and protein expression is attenuated in BMDM isolated from IκBβ$^{-/-}$ BMDM at early time points (1–6 h)

(25,26). However, it has also been reported that LPS-induced IL1β mRNA expression is not attenuated in IκBβ$^{-/-}$ BMDM at later time points (24 h) (25).

Thus, we sought to evaluate the effect of impaired and absent LPS-induced IκBβ/NFκB signaling on IL1β protein expression at later time points. Here, we demonstrate that in both AKBI and IκBβ$^{-/-}$ BMDM, LPS-induced pro-IL1β protein production was significantly attenuated at later time points (24 h, **Figure 2b**). Our results demonstrated that similar to absent IκBβ/NFκB signaling, abbreviated IκBβ/NFκB signaling is associated with attenuated expression of IL1β. These results strongly implicate a mechanistic role played by IκBβ/NFκB signaling in LPS-induced IL1β expression.

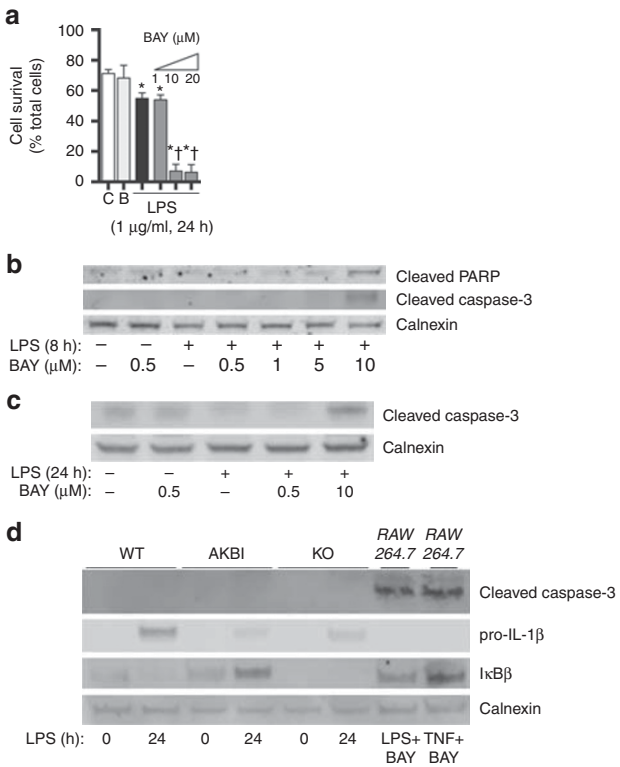

Figure 4. Targeting LPS-induced IκBβ/NFκB signaling does not cause apoptosis in macrophages. (**a**) Cell survival assessed by trypan blue exclusion in RAW 264.7 macrophages following LPS exposure (1 μg/ml, 24 h) or pretreatment with BAY 11-7085 (1–20 μM, 1 h) and LPS exposure (1 μg/ml, 24 h). Values are means+SEM from three separate experiments; h, hours; *$P<0.05$ vs. unexposed control; †, $P<0.05$ vs. LPS exposed. (**b**) Representative western blot showing cleaved PARP and cleaved caspase-3 in RAW 264.7 macrophages following LPS exposure (1 μg/ml, 8 h) or pretreatment with BAY 11-7085 (0.5–10 μM, 1 h) and LPS exposure (1 μg/ml, 8 h) with calnexin shown as loading control. (**c**) Representative western blot showing cleaved caspase-3 in RAW 264.7 macrophages following LPS exposure (1 μg/ml, 24 h) or pretreatment with BAY 11-7085 (0.5 or 10 μM, 1 h) and LPS exposure (1 μg/ml, 24 h) with calnexin shown as loading control. (**d**) Representative western blot showing cleaved caspase-3 and pro-IL-1β in WT, AKBI, and KO BMDM following LPS exposure (1 μg/ml, 0–24 h) with calnexin shown as loading control. RAW 264.7 pre-treated with BAY 11-7085 (10 μM) followed by LPS or TNF-α exposure were used as positive control for cleaved caspase-3 and lack of IL1β induction. Blot is representative of three separate experiments.

Intact IκBβ/NFκB Signaling is Not Necessary for LPS-Induced Expression of Key Anti-Apoptotic Factors

Completely inhibiting NFκB activity increases macrophage sensitivity to apoptosis induced by LPS (18) and increases apoptosis in the developing lung (35). Whether selectively targeting IκBβ/NFκB signaling to attenuate LPS-induced IL1β expression increases apoptosis in isolated macrophages or the developing lung is unknown. The anti-apoptotic NFκB target genes BCL2A1 (36), BCL-XL (37), XIAP (38), BIRC3 (39), and PAI-2 (36) are critical in preventing LPS-induced apoptosis in macrophages. Thus, we sought to determine the effect of low-dose pharmacologic IκBβ/NFκB inhibition on the LPS-induced expression of these genes. In LPS-exposed RAW 264.7 cells, pretreatment with low-dose BAY 11-7085 (between 0.5 and 1 μM) did not significantly attenuate the expression of BCL2A1, BCL-XL, XIAP, and BIRC3 genes (**Figure 3a**). Higher doses of BAY 11-7085 (5–20 μM) significantly attenuated LPS-induced expression of these anti-apoptotic genes (**Figure 3a**). Importantly, RAW 264.7 cells did not reliably express PAI-2 mRNA at baseline, so LPS-induced fold increase in expression over unexposed cells could not be determined. Thus, we normalized PAI-2 expression to LPS-induced levels. All doses of BAY 11-7085 significantly attenuated LPS-induced PAI-2 expression; however, the level of inhibition was significantly greater at doses (10–20 μM) of BAY 11-7085 where PAI-2 expression could not reliably be detected (**Figure 3a**).

Next, we assessed LPS-induced expression of BCL2A1, BCL-XL, XIAP, BIRC3, and PAI-2 genes in AKBI and IκBβ$^{-/-}$ BMDM. There was very little difference in the mRNA expression of these important anti-apoptotic genes between WT, AKBI, and IκBβ$^{-/-}$ BMDM at baseline (**Figure 3b,d**). Furthermore, there were no significant differences in the LPS-induced expression of these genes between WT (ICR) and AKBI BMDM (**Figure 3c**) or between WT (C57B6) and IκBβ$^{-/-}$ BMDM (**Figure 3d**) in LPS-induced expression of BCL2A1, BCL-XL, XIAP, BIRC3, or PAI-2 genes. These results demonstrate that LPS-induced IκBβ/NFκB signaling is not necessary for the expression of anti-apoptotic genes.

Inhibiting IκBβ/NFκB Signaling Does Not Increase Macrophage Sensitivity to Apoptosis Induced by LPS

To assess whether RAW 264.7 pretreated with low-dose BAY 11-7085 experienced increased cell death and apoptosis following exposure to LPS, we performed trypan blue exclusion assay and assessed for cleaved PARP and caspase-3 using western blot. No significant difference in cell viability at 24 h was seen between control, BAY 11-7085 treatment alone, LPS alone, or LPS pre-treated with low-dose (1 μM) BAY 11-7085 (**Figure 4a**). In contrast, LPS-exposed RAW 264.7 cells experienced >90% death when pretreated with high-dose (10–20 μM) BAY 11-7085 (**Figure 4a**). Furthermore, after 8 h of LPS exposure, cleaved PARP and caspase 3 were detected only in RAW 264.7 cells pretreated with high-dose (10 μM) BAY 11-7085 (**Figure 4b**). This finding was consistent following exposure to LPS for 24 h,

where cleaved caspase could be detected in cells pre-treated with high-dose (10 μM) BAY 11-7085 (Figure 4c). These results demonstrate that in contrast to complete inhibition of LPS-induced NFκB activity, inhibiting IκBβ/NFκB signaling does not increase macrophage sensitivity to apoptosis induced by LPS.

Additionally, we assessed WT, AKBI, and KO BMDM for cleaved caspase-3 following exposure to LPS (Figure 4d). There was no demonstrable difference in the amount of cleaved caspase-3 in LPS-exposed WT, AKBI, or IκBβ$^{-/-}$ BMDM at 24 h of exposure (Figure 4d, lanes 2, 4, 6). These results indicate that AKBI and IκBβ$^{-/-}$ BMDM are not more sensitive than WT controls to apoptosis following exposure to LPS.

Endotoxemia-Induced Pulmonary IL1β Expression is Attenuated in Newborn AKBI and IκBβ$^{-/-}$ Mice

We next assessed the effect of selectively targeting LPS-induced IκBβ/NFκB signaling on expression of IL1β and anti-apoptotic genes in the developing lung. Furthermore, to ensure that our results were not skewed by high mortality in exposed pups, we titrated the LPS exposure to determine a point of "sublethal endotoxemia," defined as >80% survival in exposed pups (Figure 5a). On the basis of these results, we exposed newborn (P0) mice to LPS (5 mg/kg, IP). Similar to what was observed in BMDM, LPS-induced pro-IL1β protein (Figure 5b) and mRNA (Figure 5c,f) expressions were significantly attenuated in the lungs of AKBI and IκBβ$^{-/-}$ neonatal mice. However, LPS-induced pulmonary expression of the anti-apoptotic factors of BCL2A1, BCL-XL, XIAP, BIRC3, and PAI-2 genes was not attenuated in either AKBI or IκBβ$^{-/-}$ mice when compared with that in controls (Figure 5d,g). Importantly, we could find no evidence of apoptosis in lungs of endotoxemic WT, AKBI, or IκBβ$^{-/-}$ neonatal mice as assessed by caspase-3 cleavage (Figure 5e,h). These results demonstrate that LPS-induced IκBβ/NFκB in the developing lung regulates IL1β expression, but does not appear necessary for the expression of key anti-apoptotic genes.

DISCUSSION

Our data clearly demonstrate that LPS-induced IκBβ/NFκB signaling can be targeted to prevent pro-inflammatory IL1β expression. We now show that LPS-induced IL1β expression is significantly inhibited in macrophages when IκBβ/NFκB signaling has been pharmacologically targeted using the IKK inhibitor BAY 11-7085, and in cells with abbreviated or absent IκBβ/NFκB signaling secondary to genetic manipulation of IκBβ expression (AKBI and IκBβ$^{-/-}$). Importantly, selective inhibition of IκBβ/NFκB signaling leaves LPS-induced expression of important anti-apoptotic proteins intact. Thus, LPS-induced IL1β expression can be attenuated without increasing susceptibility to LPS in the form of increased apoptosis. We have shown that this is true for macrophages in culture, as well as in the lung of endotoxemic neonatal animals. These findings support further study of therapeutic approaches that selectively target IκBβ/NFκB pro-inflammatory signaling, while leaving the protective component of the innate immune response intact.

It is well accepted that inflammatory injury contributes to the pathogenesis of BPD. Exposure to chorioamnionitis, early-onset, and late-onset sepsis increase the risk of developing BPD (40–42). Although postnatal steroids confer a protective anti-inflammatory effect against developing BPD, multiple detrimental off-target effects limit their clinical use. Pre-clinical studies demonstrate that global inhibition of the innate immune response has unanticipated and unwanted effects. Specifically, completely inhibiting NFκB activity exacerbates lung injury in endotoxemic neonatal mice (20,23), and increases apoptosis in LPS-exposed macrophages (18). It has recently been recognized that the nuclear activity of IκBβ contributes to sustained NFκB activity and pro-inflammatory target-gene expression (25,26). Furthermore, we have published that IκBβ/NFκB signaling can be pharmacologically targeted to attenuate the sustained expression of pro-inflammatory genes (27–30). Here we report that pulmonary expression of IL1β is reduced in endotoxemic neonatal mice with attenuated/absent IκBβ/NFκB signaling in IκBβ overexpressing (AKBI) and IκBβ$^{-/-}$ mice, whereas NFκB-regulated expression of key anti-apoptotic proteins remained intact.

Elevated levels of IL1β have been implicated in the pathogenesis of many chronic inflammatory conditions (13). Furthermore, targeting elevated IL1β has been shown to attenuate inflammation and improve outcomes in some chronic disease states (13). Multiple risk factors associated with BPD, including exposure to chorioamnionitis, sepsis (early and late onset), supplemental oxygen, and mechanical ventilation induce a chronic inflammatory state in the premature, developing lung. Importantly, multiple clinical studies have associated elevated IL1β with an increased risk of developing BPD (2,3). However, pre-clinical studies suggest that IL1β contributes to abnormal lung development after inflammatory insult, including hyperoxia, endotoxemia, and the combination of hyperoxia and endotoxemia (8–12,14–16). Furthermore, blocking IL1β activity with IL1 receptor antagonists prevents lung injury and preserves lung development after inflammatory insult (14–16). Interstingly, Liao *et al.* (16) demonstrated that following exposure to hyperoxia, the effect of attenuating the activity of IL1β at the level of inflammasome activity provides a greater protection than treatment with the IL1 receptor antagonist. Thus, there may be important differences between preventing the pro-inflammatory effects of IL1β expression through blocking its transcription or inhibiting inflammasome activity, and antagonizing the IL1 receptor. Together, these results support further study of the effects of attenuating the pro-inflammatory effects of IL1β in the developing lung.

In pre-clinical studies, attenuating IL1β activity in the developing lung has been achieved through the use of IL1 receptor antagonists (14–16). Of note, IL1α and IL1β are two distinct gene products that bind to the IL1 receptor (13). In contrast to IL1β, the effects of IL1α in the developing lung

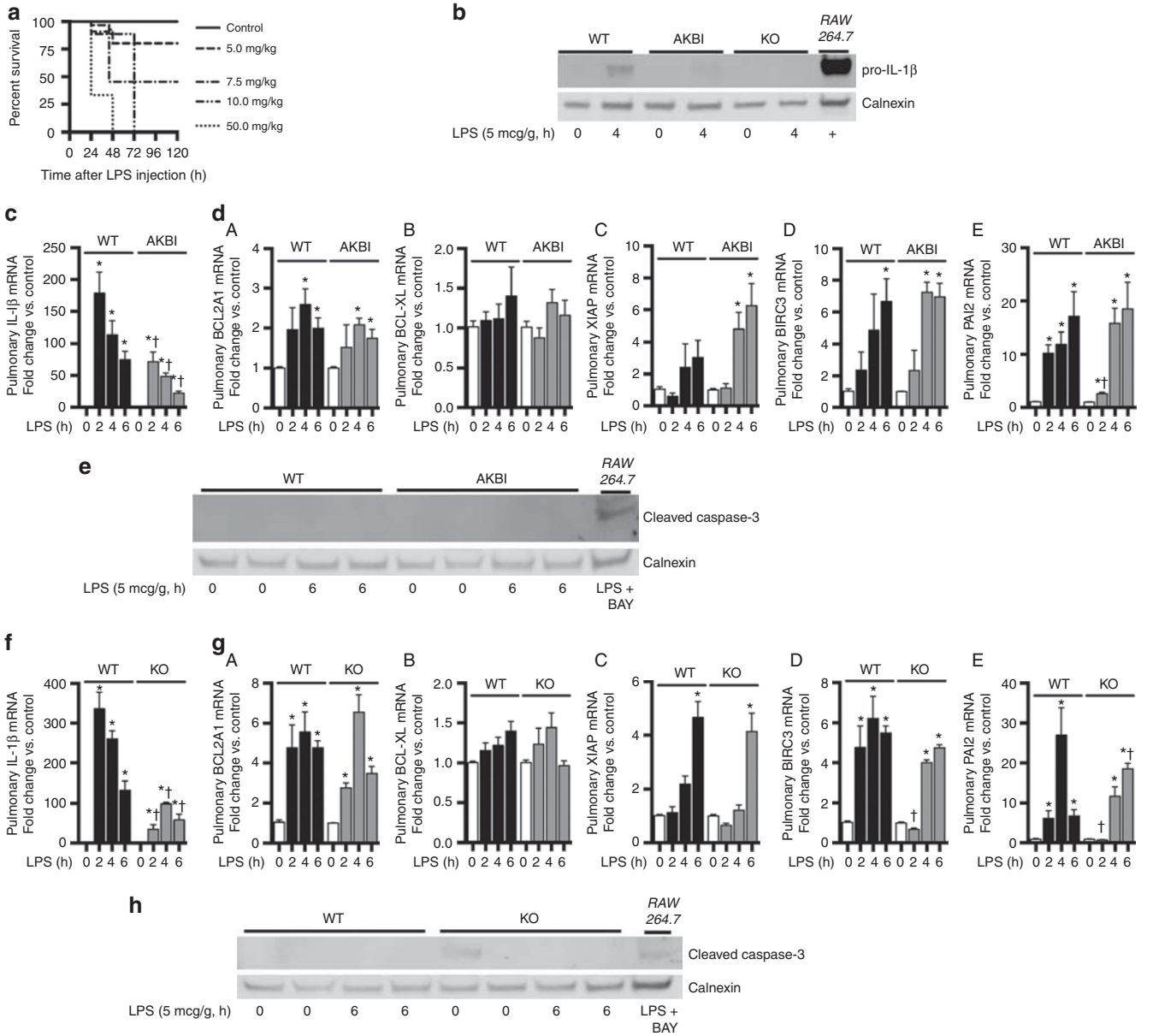

Figure 5. Targeting IκBβ/NFκB signaling does not attenuate LPS-induced expression of anti-apoptotic genes in the neonatal lung. (**a**) Percent survival of WT (ICR) mice exposed to LPS on the day of birth (0–50 mg/kg, IP). (**b**) Representative western blot showing pro-IL1β expression in WT, AKBI, and KO neonatal lung homogenate following LPS exposure (5 μg/g, 4 h). LPS-exposed RAW 264.7 cell lysate shown as positive control for IL1β. Calnexin shown as loading control. (**c**,**d**,**f**,**g**) Fold-increase in pulmonary gene expression of (**c**,**f**) IL1β and (**d**,**g**) (**A**) BCL2A1, (**B**) BCL-XL, (**C**) XIAP, (**D**) BIRC3, and (**E**) PAI2 in neonatal WT (ICR) and AKBI mice (top panels), or WT (C57B6) and KO mice (bottom panels) following LPS exposure (0–6 h, 5 mg/kg IP). Values are means+SEM ($n = 6$–8/time point, taken from three separate experiments); h, hours; *, $P < 0.05$ vs. unexposed control; $^{\dagger}P < 0.05$ vs. time-matched WT LPS exposed. (**e**,**h**) Representative western blot showing cleaved caspase-3 expression in WT, AKBI, and KO neonatal lung homogenate following LPS exposure (5 μg/g, 6 h). BAY 11-7085 pretreated, LPS-exposed RAW 264.7 cell lysate shown as positive control for caspase-3 cleavage. Calnexin shown as loading control. Images are representative of three separate experiments.

have been less well studied. However, previous studies have shown that IL1α induces surfactant production (17,43). The effect of IL1α on surfactant production appears to wane as the lung develops, suggesting that IL1α may have a unique, as yet unspecified, role in the developing lung (43). In contrast to the constitutive expression of IL1α, under normal conditions IL1β is not present intracellularly. Following stimulation, NFκB-regulated transcription must occur and this is the rate-limiting step of IL1β release (13). Given this critical step, NFκB-regulated IL1β transcription is one potential therapeutic target to prevent inflammatory injury in the developing lung.

It is well recognized that NFκB-dependent gene expression is stimulus- and cell-type-specific. Multiple layers of transcriptional control explain this observation. The IκB family of NFκB inhibitory proteins is critical in determining the

selectivity of the NFκB transcriptome. Although cytoplasmic IκBs inhibit NFκB activation, their distinct nuclear activity dictates the magnitude and duration of target gene expression. Following degradation, resynthesized IκBα and IκBβ genes enter the nucleus and act divergently. A nuclear export sequence found on IκBα allows it to export NFκB complexes from the nucleus, thus terminating NFκB activity (24). In contrast, IκBβ lacks a nuclear export sequence, and remains in the nucleus to stabilize NFκB DNA binding and sustain target gene expression (25). Thus, IκB degradation is a necessary and sufficient step regulating NFκB transcriptional activity, and the IκB isoforms play important roles in determining the profile and kinetics of the NFκB transcriptome. By understanding the key differences between IκBα and IκBβ, we may be able to identify a therapeutic target to attenuate the expression of pro-inflammatory NFκB target genes, leaving the expression of anti-inflammatory and anti-apoptotic genes intact.

The current report has a number of limitations. We have shown that *in vitro* low-dose pharmacologic IKK inhibition prevents IκBβ/NFκB degradation. In contrast, at these low doses, LPS-induced IκBα degradation occurs. We were not able to pharmacologically inhibit IκBβ/NFκB signaling *in vivo*. Further studies are necessary to determine whether IκBβ/NFκB signaling can be specifically targeted at the IKK–IκBβ interface *in vivo*. Previous groups have demonstrated that IκBβ$^{-/-}$ have abbreviated LPS-induced NFκB activity due to the absence of nuclear IκBβ activity (25,26). Similarly, we have previously demonstrated that sustained LPS-induced NFκB activity is prevented by IκBβ overexpression (28). However, in addition to IκBβ overexpression, the AKBI mice express no IκBα. (28) Thus, in these mice, findings may be explained by the absence of IκBα/NFκB signaling, rather than attenuated IκBβ/NFκB signaling. The current report focused on the transcriptional regulation of LPS-induced IL1β expression. We did not evaluate inflammasome activation/activity, or assess systemic levels of mature IL1β. Further studies are necessary to determine whether targeting IκBβ/NFκB transcriptional regulation of IL1β affects inflammasome activity and levels of mature IL1β, and to determine how this affects the response to endotoxemia and lung development in neonatal animals. Furthermore, we evaluated the pulmonary expression of key NFκB-regulated apoptotic genes. Undoubtedly, the LPS-induced expression of other genes differs between WT, IκBβ$^{-/-}$, and AKBI mice. Further studies are necessary to determine the ultimate effect of altering the LPS-induced NFκB transcriptome on the developing lung, and perhaps more importantly, whether these changes affect neonatal mortality following endotoxemia.

We conclude that it may be reasonable to identify methods to specifically target LPS-induced IκBβ/NFκB signaling while leaving IκBα/NFκB signaling intact. Targeting this signaling in the developing lung attenuates the expression of IL1β, and may prevent lung injury associated with early inflammatory insult. We speculate that further study of IκBβ/NFκB signaling may reveal therapeutic targets to prevent the chronic inflammatory injury that contributes to the pathogenesis of BPD.

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