ARTICLE

# CUL4B negatively regulates Toll-like receptor-triggered proinflammatory responses by repressing *Pten* transcription

Yu Song[1], Peishan Li[1], Liping Qin[1], Zhiliang Xu[1], Baichun Jiang[1], Chunhong Ma[2], Changshun Shao[3] and Yaoqin Gong[1]

Toll-like receptors (TLRs) play critical roles in innate immunity and inflammation. The molecular mechanisms by which TLR signaling is fine-tuned remain to be completely elucidated. Cullin 4B (CUL4B), which assembles the CUL4B-RING E3 ligase complex (CRL4B), has been shown to regulate diverse developmental and physiological processes by catalyzing monoubiquitination for histone modification or polyubiquitination for proteasomal degradation. Here, we identified the role of CUL4B as an intrinsic negative regulator of the TLR-triggered inflammatory response. Deletion of CUL4B in macrophages increased the production of proinflammatory cytokines and decreased anti-inflammatory cytokine IL-10 production in response to pathogens that activate TLR3, TLR4, or TLR2. Myeloid cell-specific *Cul4b* knockout mice were more susceptible to septic shock when challenged with lipopolysaccharide, polyinosinic-polycytidylic acid or *Salmonella typhimurium* infection. We further demonstrated that enhanced TLR-induced inflammatory responses in the absence of CUL4B were mediated by increased GSK3β activity. Suppression of GSK3β activity efficiently blocked the TLR-triggered increase in proinflammatory cytokine production and attenuated TLR-triggered death in *Cul4b* mutant mice. Mechanistically, CUL4B was found to negatively regulate TLR-triggered signaling by epigenetically repressing the transcription of *Pten*, thus maintaining the anti-inflammatory PI3K-AKT-GSK3β pathway. The upregulation of PTEN caused by CUL4B deletion led to uncontrolled GSK3β activity and excessive inflammatory immune responses. Thus, our findings indicate that CUL4B functions to restrict TLR-triggered inflammatory responses through regulating the AKT-GSK3β pathway.

**Keywords:** CUL4B; TLR signaling; Transcriptional repression

*Cellular & Molecular Immunology* (2021) 18:339–349; https://doi.org/10.1038/s41423-019-0323-0

## INTRODUCTION

Toll-like receptors (TRLs) are the primary sensors that detect conserved molecular patterns on microorganisms and thus act as important components of innate immunity against invading pathogens.[1,2] To date, 13 TLRs (TLR1-TLR13) in humans and mice have been identified.[3–5] By recognizing conserved pathogen components, TLRs recruit the adaptor proteins myeloid differentiation primary response 88 (MyD88) and/or TIR domain-containing adaptor-inducing interferon–β (TRIF) and activate multiple pathways to induce pathogen elimination by immune mediators.[3,6–8] Although TLR activation is important for host defense, excessive immune and inflammatory responses cause tissue damage and serious diseases, such as septic shock. Thus, the restriction of TLR signaling is essential to avoid uncontrolled inflammatory immune responses and maintain immune homeostasis.

Glycogen synthase kinase 3β (GSK3β) serves as the node of convergent signaling pathways that regulate a variety of cellular processes, such as metabolism, cell proliferation, differentiation, and development.[9–11] Unlike most kinases, GSK3β is constitutively active in resting cells, and its activity can be inhibited through N-terminal serine phosphorylation (ser 9) by the PI3K pathway.[9,12,13] Recently, GSK3β was identified as a key mediator of proinflammatory cytokine production in diverse TLR signaling pathways.[12,14,15] The inhibition of GSK3β potently suppressed the production of proinflammatory cytokines and concurrently augmented the production of anti-inflammatory IL-10 in response to several TLR signaling pathways.[16–18] Although these studies clearly documented the importance of GSK3β in TLR-mediated cytokine production, little is known about how GSK3β activity is regulated during TLR-mediated immune responses.

Cullin 4B (CUL4B) functions as a scaffold protein in the Cullin-4B-Ring E3 ligase complex (CRL4B) and participates in regulating diverse physiologically and developmentally controlled processes by targeting specific substrates for ubiquitin-dependent degradation or modification.[19–21] Mutations in human *CUL4B* are a common cause of X-linked mental retardation syndrome.[22–27] In addition to being mentally retarded, patients with *CUL4B* mutations also manifest short stature, the absence of speech, an elevated monocyte count, and other developmental defects.[23–26] The lack of CUL4B in mice markedly compromised extraembryonic development,[28,29] hematopoiesis,[30] neurogenesis,[31,32] and adipogenesis.[33] Mechanistically, CRL4B can catalyze either polyubiquitination for proteasomal degradation[33–36] or the monoubiquitination of H2A for epigenetic modification.[37] Recently, we showed that the deletion of CUL4B in the hematopoietic system led to the aberrant accumulation of myeloid-derived suppressive cells (MDSCs)

[1]Key Laboratory of Experimental Teratology, Ministry of Education, Institute of Molecular Medicine and Genetics, School of Basic Medical Sciences, Shandong University, Jinan, Shandong, China; [2]Key Laboratory of Experimental Teratology, Ministry of Education, Department of Immunology, School of Basic Medical Science, Shandong University, Jinan, Shandong, China and [3]State Key Laboratory of Radiation Medicine and Protection, Institutes for Translational Medicine, Soochow University, Suzhou, Jiangsu, China
Correspondence: Yaoqin Gong (yxg8@sdu.edu.cn)

Received: 29 July 2019 Accepted: 17 October 2019
Published online: 15 November 2019

mediated by downregulation of the AKT/β-catenin pathway.[30] CUL4B was shown to sustain the AKT/β-catenin pathway by repressing the phosphatases PP2A and PHLPP1/2, which dephosphorylate and inactive AKT.[30] However, the role of CUL4B in TLR-mediated innate immune responses remains unknown. In this study, we demonstrated that CUL4B functions as a negative regulator of TLR4/3/2-mediated immune and inflammatory responses via epigenetically repressing *Pten* transcription, thus restraining GSK3β activity. The depletion of CUL4B in macrophages augmented TLR4-mediated, TLR3-mediated, and TLR2-mediated proinflammatory cytokine production and decreased production of the anti-inflammatory cytokine IL-10. Myeloid cell-specific *Cul4b* knockout (MKO) mice exhibited an increased susceptibility to septic shock relative to wild-type (WT) control mice when challenged with lipopolysaccharide (LPS), polyinosinic-polycytidylic acid [poly(I:C)] or *Salmonella typhimurium* infection. The inhibition of GSK3β activity efficiently blocked the increase in TLR-triggered proinflammatory cytokine production and attenuated TLR-triggered death caused by *Cul4b* deletion. These findings not only provide mechanistic insight into the role of CUL4B in regulating innate immune responses but also suggest GSK3β as a target for sepsis therapy.

## RESULTS

### Deletion of CUL4B in macrophages increases lipopolysaccharide-induced production of proinflammatory cytokines

To investigate the role of CUL4B in innate immune and inflammatory responses, we generated myeloid-specific *Cul4b* knockout mice (referred to as MKO mice) by crossing *Cul4b*-floxed mice with LysM-driven transgenic mice.[28] We examined the protein expression of CUL4B in peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMDMs) and confirmed that CUL4B was effectively deleted in macrophages (Fig. 1a). Consistent with our previous observations in hematopoietic-specific *Cul4b* knockout mice, the MKO mice exhibited normal growth and survival and produced macrophages, neutrophils, and dendritic cells in bone marrow and spleens at frequencies comparable to those in wild-type mice (Fig. 1b and Suppl Fig. S1). To determine the role of CUL4B in regulating the LPS-induced inflammatory response, BMDMs were prepared from *Cul4b* MKO and control mice and exposed to the TLR4 agonist LPS, and the production of proinflammatory cytokines was determined. As shown in Fig. 1c, the mRNA levels of *Tnfα*, *Il1β*, and *Il6* were significantly increased in CUL4B-deficient BMDMs treated with LPS compared to their WT counterparts. Consistent with their mRNA expression, ELISA showed that deletion of CUL4B significantly increased the secretion of these proinflammatory cytokines (Fig. 1d). These results suggest that CUL4B negatively regulates the TLR4-mediated production of proinflammatory cytokines.

### Deletion of CUL4B in myeloid cells aggravates LPS-induced or gram-negative bacterium-induced septic shock

To determine the role of CUL4B in the TLR4-mediated immune response in vivo, age-matched and sex-matched WT and MKO mice were intraperitoneally injected with LPS, and the serum levels of proinflammatory cytokines were measured. We found that the serum levels of TNF-α, IL-6, and IL-1β were higher in *Cul4b* MKO mice than in control mice (Fig. 2a). Consistent with changes in cytokine production in the sera, *Cul4b* MKO mice died earlier and had lower survival rates than control mice when challenged with LPS (Fig. 2b). To confirm this phenotype, we also used the gram-negative bacterium *S. typhimurium* to induce sepsis in WT and MKO mice. Upon *S. typhimurium* infection, MKO mice also produced more proinflammatory cytokines (Fig. 2c) and exhibited a shorter survival time (Fig. 2d), as well as a higher bacterial load in the blood (Fig. 2e) than their WT counterparts, which is similar to the results of published reports showing that proinflammatory cytokines promote the dissemination of *E. coli*.[38,39] Furthermore, we observed more severe lung injury in MKO mice, as characterized by more immune cell infiltration after challenge with LPS or *S. typhimurium* (Fig. 2f and Suppl Fig. S2). These data indicate that the deletion of CUL4B aggravated LPS-induced or bacteria-induced septic shock, further supporting the notion that CUL4B negatively regulates innate inflammatory responses.

### Increased production of proinflammatory cytokines in *Cul4b*-deficient macrophages is mediated by enhanced GSK3β activity

GSK3β functions as a negative regulator of the TLR4-mediated inflammatory response.[16] Our recent study showed that CUL4B can regulate GSK3β activity.[30,40] Thus, we next examined the effect of CUL4B ablation on the activity of GSK3β in BMDMs. We observed that the levels of GSK3β phosphorylated at ser9, reflecting GSK3β inactivation, were significantly reduced in LPS-stimulated *Cul4b*-deficient BMDMs (Fig. 3a), indicating that the increased kinase activity of GSK3β could be responsible for the increased TLR4-mediated cytokine induction caused by CUL4B deletion. Indeed, pretreatment with the GSK3β inhibitor SB216763 efficiently blocked increased TLR4-mediated cytokine production in *Cul4b*-deficient BMDMs (Fig. 3b). Furthermore, the ability of SB216763 to decrease the TLR4-triggered production of proinflammatory cytokines was also demonstrated in MKO mice in vivo. As shown in Fig. 3c, d, SB216763 efficiently blocked increased proinflammatory cytokine production and increased the survival rate of LPS-challenged MKO mice. Taken together, these results indicate that increased GSK3β activity may mediate the enhanced production of TLR4-triggered proinflammatory cytokines in *Cul4b* MKO mice, suggesting that GSK3β is one of the downstream effectors of CUL4B in response to LPS stimulation or gram-negative bacterial infection.

### Lack of CUL4B potentiates TLR-3-mediated and TLR2-mediated immune responses

In addition to regulating TLR4, GSK3β is critically involved in immune responses triggered by other TLRs.[15–17,41,42] We therefore evaluated the role of CUL4B in TLR3-induced, TLR2-induced, or TLR7/8–induced inflammatory responses. We stimulated WT and *Cul4b*-deficient BMDMs with poly(I:C) (a ligand of TLR3), R848 (a ligand of TLR7 and TLR8), or PGN (a ligand of TLR2) and then examined the transcription of proinflammatory cytokines. We found that CUL4B deficiency potentiated poly(I:C)-induced or PGN-induced transcription of *Tnfa, Il1β, and Il6*, but not that induced by R848 (Fig. 4a). Consistently, poly(I:C)-induced or PGN-induced secretion of TNF-α, IL-1β, and IL-6, but not that induced by R848, was significantly increased in *Cul4b*-deficient BMDMs (Fig. 4b). To further confirm that TLR7/8 signaling is not affected by CUL4B deficiency, we tested another agonist of TLR7/8, CL097, and obtained similar results (Suppl Fig. 3). We then challenged MKO mice with poly(I:C) in vivo. Compared to WT mice, *Cul4b*-deficient mice produced significantly higher levels of IL-6, TNF-α, and IFN-β in the serum and developed a more severe innate inflammatory response after being challenged with poly(I:C) plus D-galactosamine (Fig. 4c, d). Similar to the TLR4-triggered response, the level of GSK3β phosphorylated at serine 9 was decreased in TLR3-triggered or TLR2-triggered MKO BMDMs (Fig. 4e, f). The administration of SB216763 efficiently blocked the increased proinflammatory cytokine production and rescued the reduced survival caused by CUL4B depletion (Fig. 4g, h). Together, these results suggest that CUL4B also negatively regulates TLR3-mediated and TLR2-mediated inflammatory responses via regulating GSK3β activity.

### CUL4B restricts NF-κB but promotes CREB signaling in macrophages

GSK3β was reported to positively regulate LPS-induced NF-κB activity, which controls proinflammatory immune responses, and

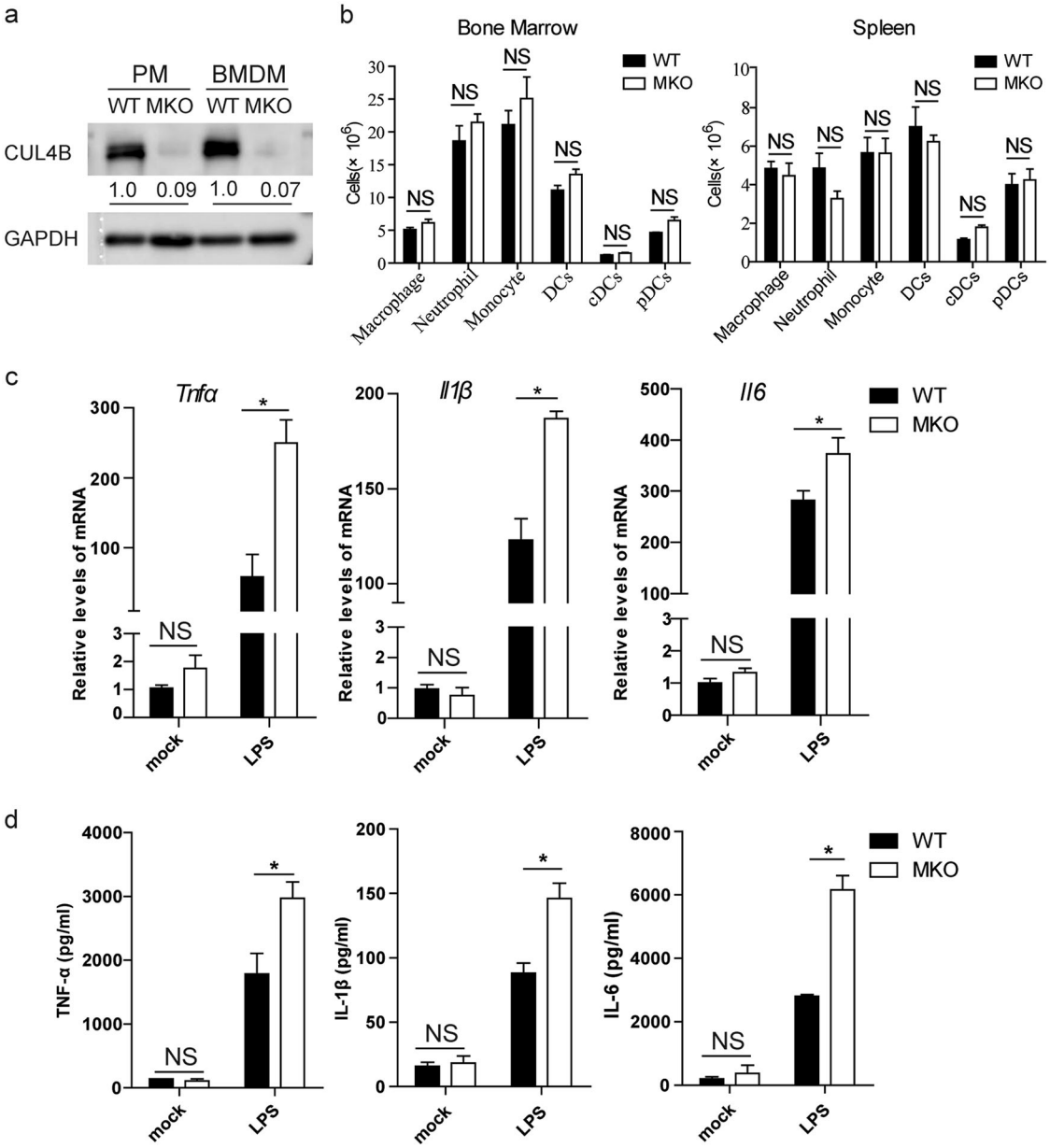


**Fig. 1** Deletion of CUL4B in macrophages increases TLR4-mediated production of proinflammatory cytokines. **a** CUL4B levels in peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMDMs). PMs and BMDMs were prepared from WT and MKO mice, and the CUL4B level was determined by immunoblot analysis. Band intensities given underneath gel images were determined using ImageJ software and are presented as the fold change. **b** Numbers of macrophages, neutrophils, monocytes, dendritic cells (DCs), conventional dendritic cells (cDCs), and plasmacytoid dendritic cells (pDCs) in bone marrow (left) and spleens (right) from WT and MKO mice determined by flow cytometry. **c** Effects of CUL4B deficiency on LPS-induced transcription of *Tnfa*, *Il1β*, and *Il6* in BMDMs. WT and MKO BMDMs were stimulated with LPS (100 ng/ml) for 2 h before qRT-PCR was performed. **d** Effects of CUL4B deficiency on LPS-induced production of TNF-α, IL-1β, and IL-6 in BMDMs. WT and MKO BMDMs were stimulated with LPS (100 ng/ml) for 3 h, and then the concentrations of TNF-α, IL-1β, and IL-6 in the supernatant were determined by ELISA. The data in **b** are presented as the mean ± SEM. The data in **c** and **d** are presented as the mean ± SD. Data are representative of more than three independent experiments. NS not significant; $*p < 0.05$, $**p < 0.01$

suppress the activity of CREB, a critical component for anti-inflammatory cytokine IL-10 production.[16] To further confirm that CUL4B regulates the inflammatory response, at least in part, by suppressing GSK3β activity, we next examined the key molecules in these signaling pathways. As expected, we observed the enhanced phosphorylation of IκB and NF-κB p65 in LPS-treated, poly(I:C)-treated, or PGN-treated *Cul4b*-deficient macrophages (Fig. 5a). Consistently, NF-kB activation was significantly increased in LPS-stimulated, poly(I:C)-stimulated, or PGN-stimulated *Cul4b*-deficient macrophages, as determined by transcription factor activity assays (Fig. 5b). In contrast, the levels of phosphorylated CREB and CREB activity were reduced in LPS-triggered, poly(I:C)-triggered, or PGN-triggered *Cul4b*-deficient macrophages (Fig. 5a, c). Correspondently, the level of IL-10 production was reduced in LPS-stimulated, poly(I:C)-stimulated, or PGN-stimulated *Cul4b*-deficient macrophages (Fig. 5d). These results demonstrate that CUL4B plays a regulatory role in the TLR4/3/2-mediated inflammatory response by suppressing the production of proinflammatory cytokines and promoting anti-inflammatory cytokine production.

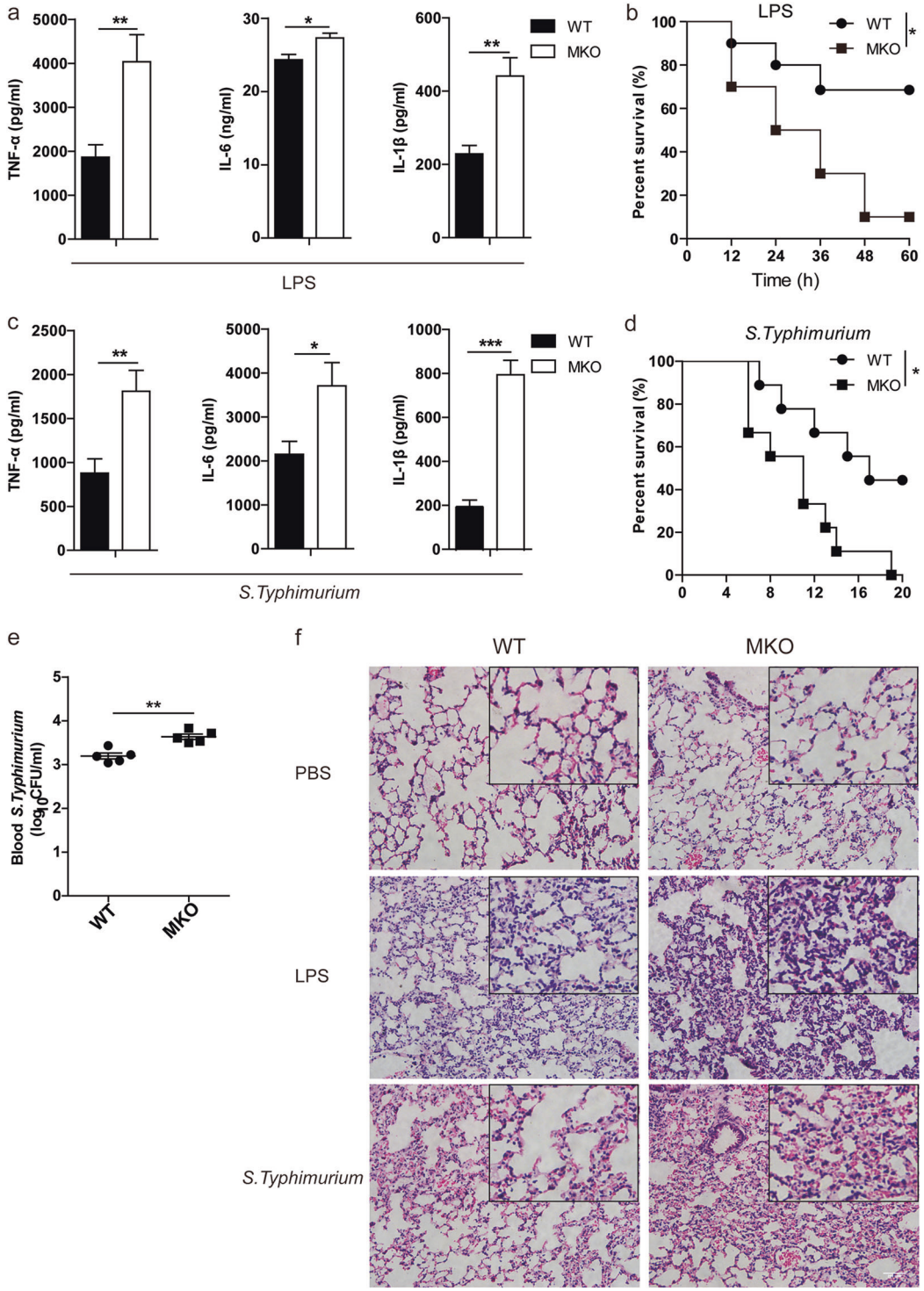


**Fig. 2** Deletion of CUL4B in myeloid cells aggravates LPS-induced or gram-negative bacterium-induced septic shock. **a** Effects of CUL4B deficiency on LPS-induced cytokine production in serum. Sex-matched and age-matched WT and MKO mice ($n = 5$ for each group) were intraperitoneally injected with LPS (10 μg/g) for 4 h. The serum concentrations of TNF-α, IL-1β, and IL-6 were determined by ELISA. **b** Effects of CUL4B deficiency on LPS-induced inflammatory death. WT and MKO mice ($n = 10$ for each group) were intraperitoneally injected with LPS (10 μg/g). Mouse survival was monitored over the following 60 h. **c** Effects of CUL4B deficiency on *S. typhimurium*-induced cytokine production in serum. WT and MKO mice ($n = 5$ for each group) were intravenously administrated *S. typhimurium* ($5 \times 10^4$ CFU/ml) for 6 h. Sera were collected, and the concentrations of TNF-α, IL-1β, and IL-6 were determined by ELISA. **d** Effects of CUL4B deficiency on *S. typhimurium*-induced septic shock. WT and MKO mice ($n = 9$ for each group) were infected with *S. typhimurium* ($5 \times 10^4$ CFU/ml), and survival was monitored every day for 20 days. **e** Effects of CUL4B deficiency on bacterial load in the blood. WT and MKO mice ($n = 5$ per genotype) were administrated *S. typhimurium* ($5 \times 10^4$ CFU/ml) for 24 h. The bacterial load was determined. **f** Effects of CUL4B deficiency on LPS-induced or *S. typhimurium*-induced lung injury. Hematoxylin and eosin staining of lung sections from WT and MKO mice after challenge with PBS, LPS or *S. typhimurium* for 6 h. Original magnification is ×200. Scale bars, 200 μm. The data in **a**, **c** and **e** are presented as the mean ± SEM. Data are representative of more than three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

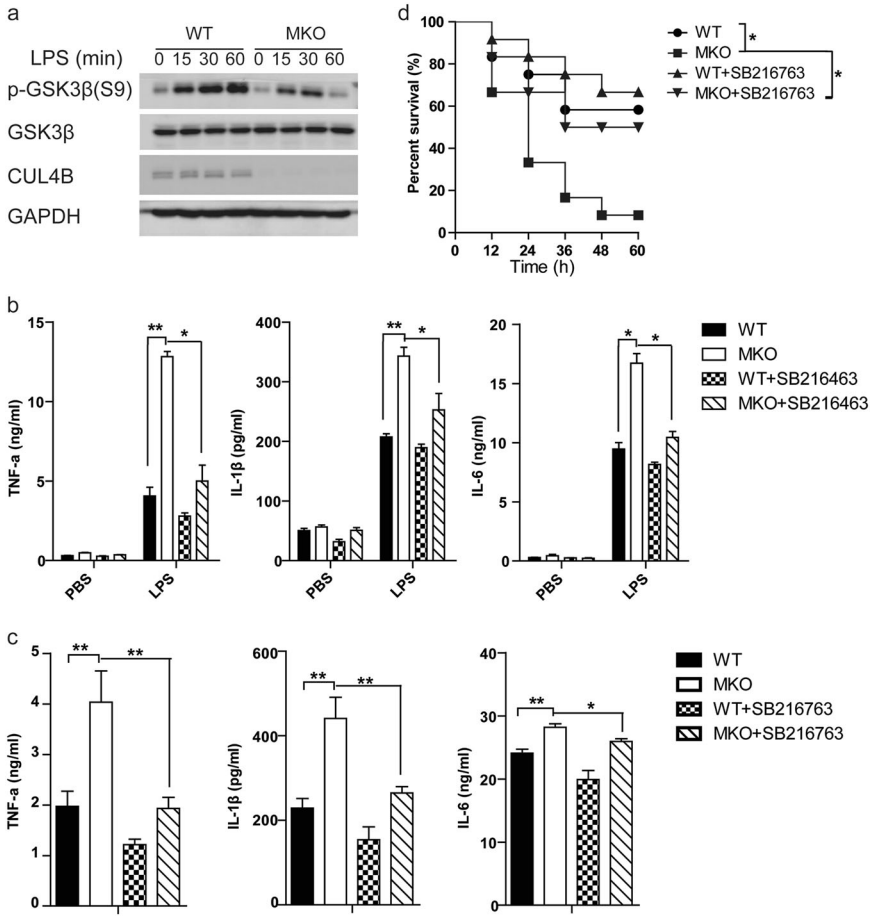


**Fig. 3** Increased production of proinflammatory cytokines in *Cul4b*-deficient macrophages is mediated by enhanced GSK3β activity. **a** Effects of CUL4B deficiency on LPS-induced phosphorylation of GSK3β (Ser 9) in BMDMs. WT and MKO BMDMs were stimulated with LPS (100 ng/ml) for the indicated times before immunoblotting with the indicated antibodies. **b** Negative regulation of LPS-induced cytokine production in BMDMs with the GSK3β inhibitor SB216763. WT and MKO BMDMs were pretreated with or without SB216763 (10 μM) for 1 h and stimulated with LPS (100 ng/ml) for 3 h. The concentrations of TNF-α, IL-1β, and IL-6 in the medium were measured by ELISA. **c** Inhibition of LPS-induced cytokine production in the serum. WT and MKO mice ($n = 10$ for each group) were pretreated with or without the GSK3β inhibitor SB216763 (25 μg/g) for 2 h and then administered LPS (10 μg/g) for 3 h. Sera were collected to measure the TNF-α, IL-1β, and IL-6 levels by ELISA. **d** Inhibition of LPS-induced death with the GSK3β inhibitor SB216763. WT and MKO mice ($n = 10$ for each group) pretreated with or without the GSK3β inhibitor SB216763 (25 μg/g) for 1 h were injected with LPS (10 μg/g, intraperitoneal) and then monitored for survival. The data are presented as the mean ± SD in **b** and as the mean ± SEM in **c**. Data are representative of more than three independent experiments. *$P < 0.05$; **$P < 0.01$

Transcriptional upregulation of *Pten* contributes to enhanced GSK3β activity in CUL4B-deficient macrophages

As serine 9 of GSK3β is phosphorylated by AKT, we next determined the level of AKT phosphorylated at Thr308 and Ser473, which indicates maximal AKT activity. We found that the levels of phosphorylated AKT were significantly decreased in LPS-stimulated, poly(I:C)-stimulated, or PGN-stimulated *Cul4b*-deficient macrophages (Fig. 6a–c), indicating that the decreased GSK3β phosphorylation at ser9 is due to decreased AKT kinase activity. This decrease in the level of AKT phosphorylation could be attributed to the decreased activities of kinases upstream of AKT or increased activities of phosphatases that dephosphorylate AKT. While no significant difference in the levels of AKT kinases and phosphatases was detected (Suppl Fig. S4), PTEN, a negative regulator of AKT, was found to be significantly elevated in *Cul4b*-deficient macrophages (Fig. 6a–c).

Our previous studies showed that CUL4B functions as a transcriptional corepressor.[37,43] We thus next examined the mRNA level of *Pten*. Quantitative real-time PCR revealed that the *Pten* mRNA level was significantly higher in *Cul4b*-deficient BMDMs (Fig. 6d). To further elucidate the molecular mechanism by which CUL4B inhibits PTEN expression, we used the chromatin immunoprecipitation (ChIP) assay using primer pairs specific for a region of approximately 70 bp to 1500 bp upstream of the *Pten* transcription initiation site to confirm whether the *Pten* gene is bound by the CUL4B complex. The ChIP assay indicated that CUL4B binds the *Pten* promoter from the −1487 to −1344 region (Fig. 6e). Importantly, we detected decreased binding of CUL4B to the *Pten* promoter during TLR-triggered responses, which is consistent with increased *Pten* expression during TLR agonist stimulation (Fig. 6f). Notably, EZH2, H2AK119ub1, and H3K27me3 were also bound to the same region (Fig. 6g). To determine the effect of CUL4B deletion on the levels of CUL4B, EZH2, and H2AK119ub1 at the *Pten* promoter, quantitative ChIP assays were performed in CUL4B-null and control macrophages with antibodies specific for CUL4B, H2AK119ub1, EZH2, H3K27me3, and H3K4me3. While CUL4B, EZH2, H2AK119ub1, and H3K27me3 were enriched at the promoter of the *Pten* gene in cells in the control group, CUL4B deletion significantly decreased their occupancy at the promoter. Meanwhile, the levels of H2AK119ub1 and H3K27me3 at the *Pten* promoter were also significantly reduced, while the level of H3K4me3 on the *Pten* promoter was increased

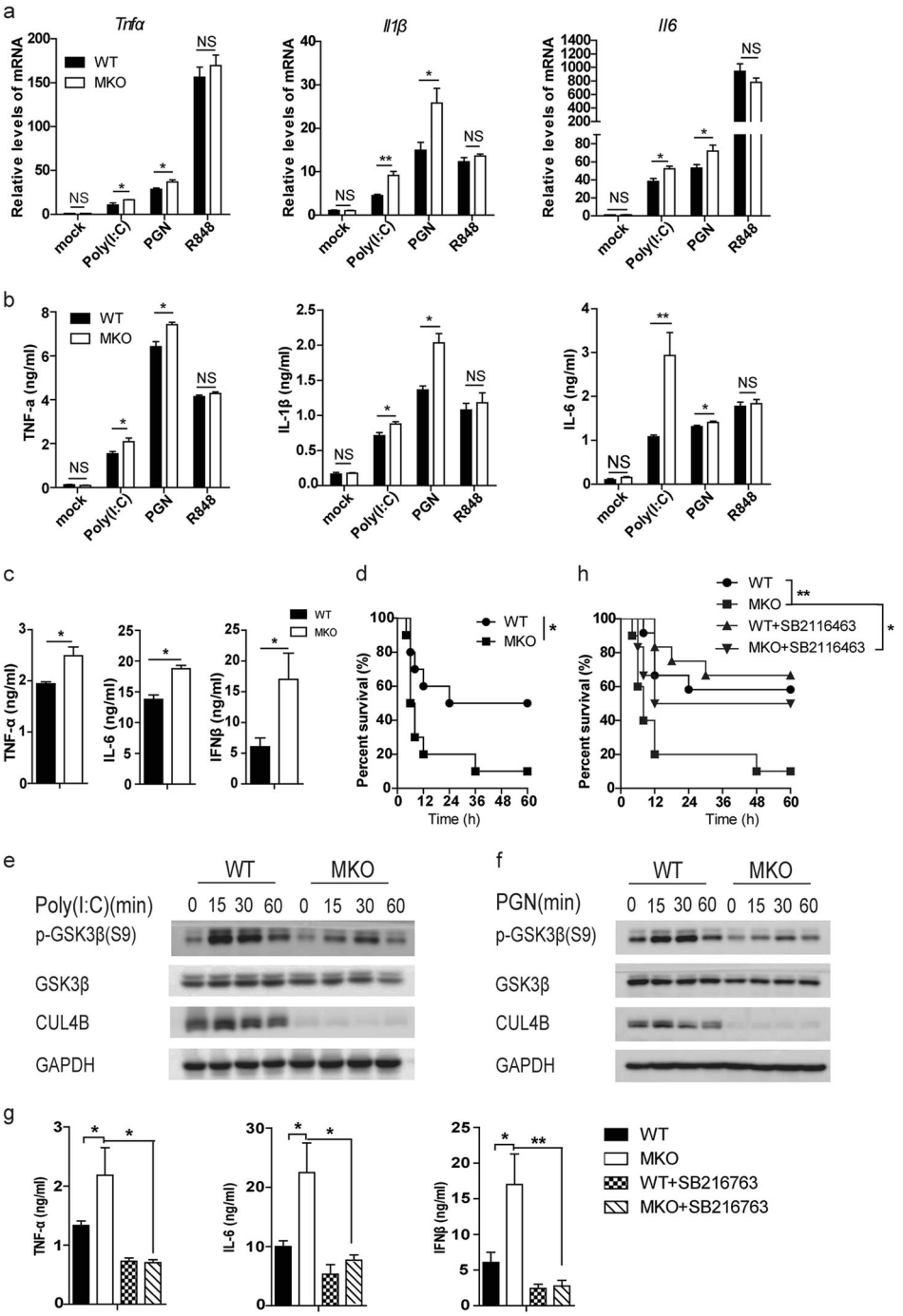


**Fig. 4** CUL4B deficiency potentiates TLR2-mediated and TLR3-mediated immune responses. **a** Effects of CUL4B deficiency on TLR-triggered transcription of *Tnfa, Il1β*, and *Il6* in BMDMs. WT and MKO BMDMs were stimulated with PGN (20 μg/ml), poly(I:C) (50 μg/ml), or R848 (20 nM) for 2 h before qRT-PCR was performed. **b** Effects of CUL4B deficiency on PGN-induced, poly(I:C)-induced and R848-induced production of TNF-α, IL-6, and IL-1β in BMDMs. WT and MKO BMDMs were subjected to the indicated stimulation for 3 h. The concentrations of TNF-α, IL-6, and IL-1β in the supernatant were determined by ELISA. **c** Effects of CUL4B deficiency on poly(I:C)-induced cytokine production in the serum. WT and MKO mice ($n = 5$ for each group) were administered poly(I:C) (5 μg/g) for 4 h, and the concentrations of TNF-α, IL-6, and IFNβ in the serum were measured by ELISA. **d** Effects of CUL4B deficiency on poly(I:C)-induced inflammatory death. Sex-matched and age-matched WT and MKO mice ($n = 11$ for each group) were i.p. injected with poly(I:C) (5 μg/g) and monitored for survival. **e–f** Effects of CUL4B deficiency on (**e**) poly(I:C)-induced and (**f**) PGN-induced phosphorylation of GSK3β (Ser 9) in BMDMs. WT and MKO BMDMs were stimulated with poly(I:C) (50 μg/ml) or PGN (20 μg/ml) for the indicated times before immunoblotting with the indicated antibodies. **g** Inhibition of poly(I:C)-induced cytokine production in the serum. WT and MKO mice ($n = 5$ for each group) were pretreated with or without SB216763 (25 μg/g) for 1 h and then stimulated with poly(I:C) (5 μg/g) for 4 h. Levels of TNF-α, IL-6, and IFN-β in the plasma were determined by ELISA. **h** Inhibition of poly(I:C)-induced death with the GSK3β inhibitor SB216763. WT and MKO mice ($n = 11$ per group) were pretreated with the GSK3β inhibitor SB216763 (25 μg/g) for 1 h and then administered poly(I:C) (5 μg/g). Mouse survival was monitored. The data are presented as the mean ± SD in **a** and **b** and as the mean ± SEM in **c** and **g**. Data are representative of more than three independent experiments. NS not significant; $^*P < 0.05$; $^{**}P < 0.01$

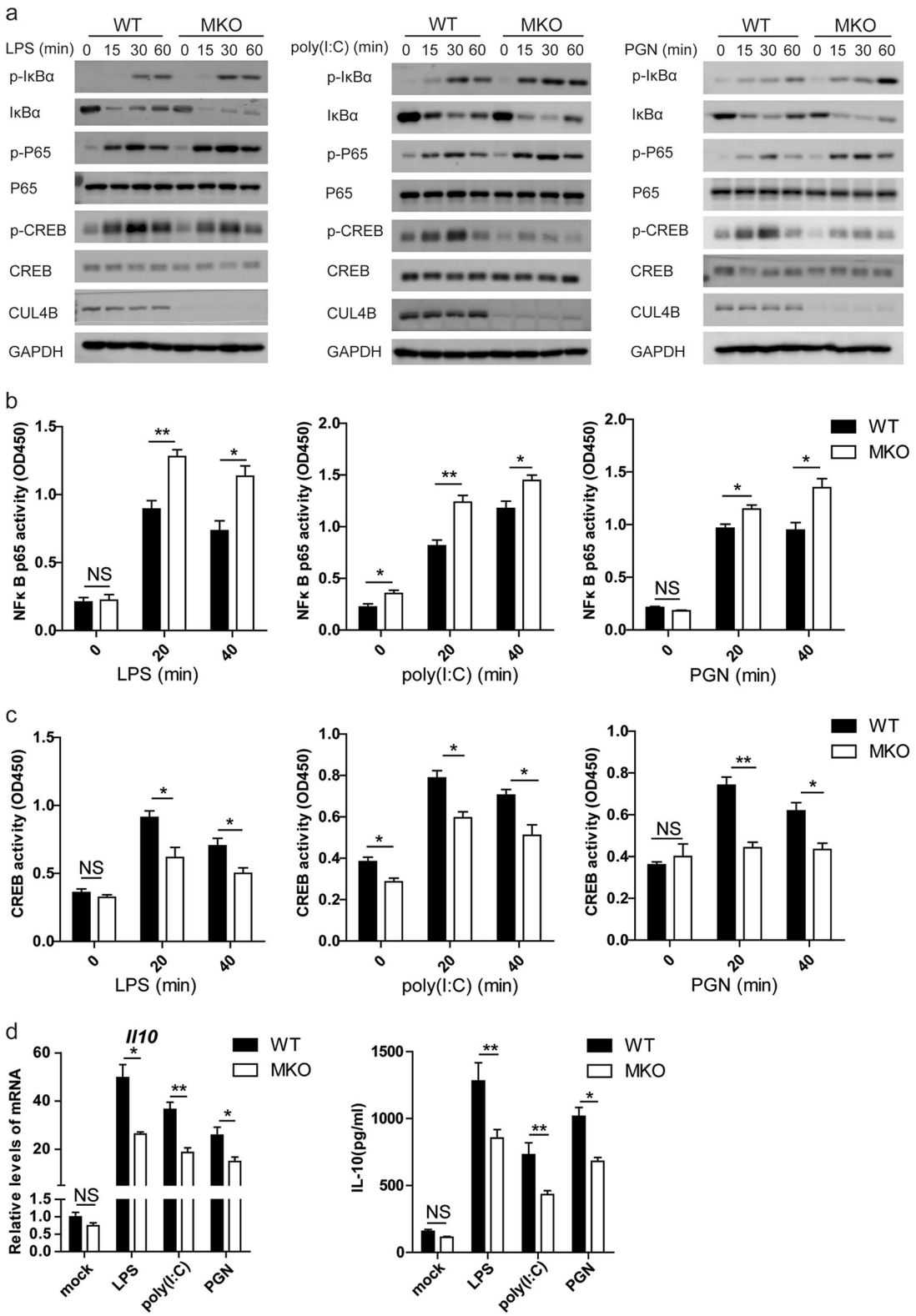


**Fig. 5** Lack of CUL4B in macrophages results in upregulated NFκB and downregulated CREB signaling. **a** Effects of CUL4B deficiency on LPS-induced, poly(I:C)-induced and PGN-induced phosphorylation of IκBα, P65, and CREB in BMDMs. WT and MKO BMDMs were stimulated with LPS (100 ng/ml), poly(I:C) (50 μg/ml), or PGN (20 μg/ml) for the indicated times before immunoblotting was performed with the indicated antibodies. **b–c** Effect of CUL4B deficiency on the transcriptional activity of NF-κB and CREB by TLR agonists. WT and MKO BMDMs were stimulated with LPS (100 ng/ml), poly(I:C) (50 μg/ml), or PGN (20 μg/ml) for 20 or 40 min. Equal amounts of nuclear extracts were analyzed for NF-κB (**b**) and CREB (**c**) activation using an ELISA-based chemiluminescent kit as described in the Materials and methods section. **d** Effects of CUL4B deficiency on TLR-triggered IL-10 expression in BMDMs. WT and MKO BMDMs were stimulated with LPS (100 ng/ml), poly(I:C) (50 μg/ml) or PGN (20 μg/ml) for 2 h before qRT-PCR or ELISA was performed. The data are presented as the mean ± SD. Data are representative of more than three independent experiments. NS, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$

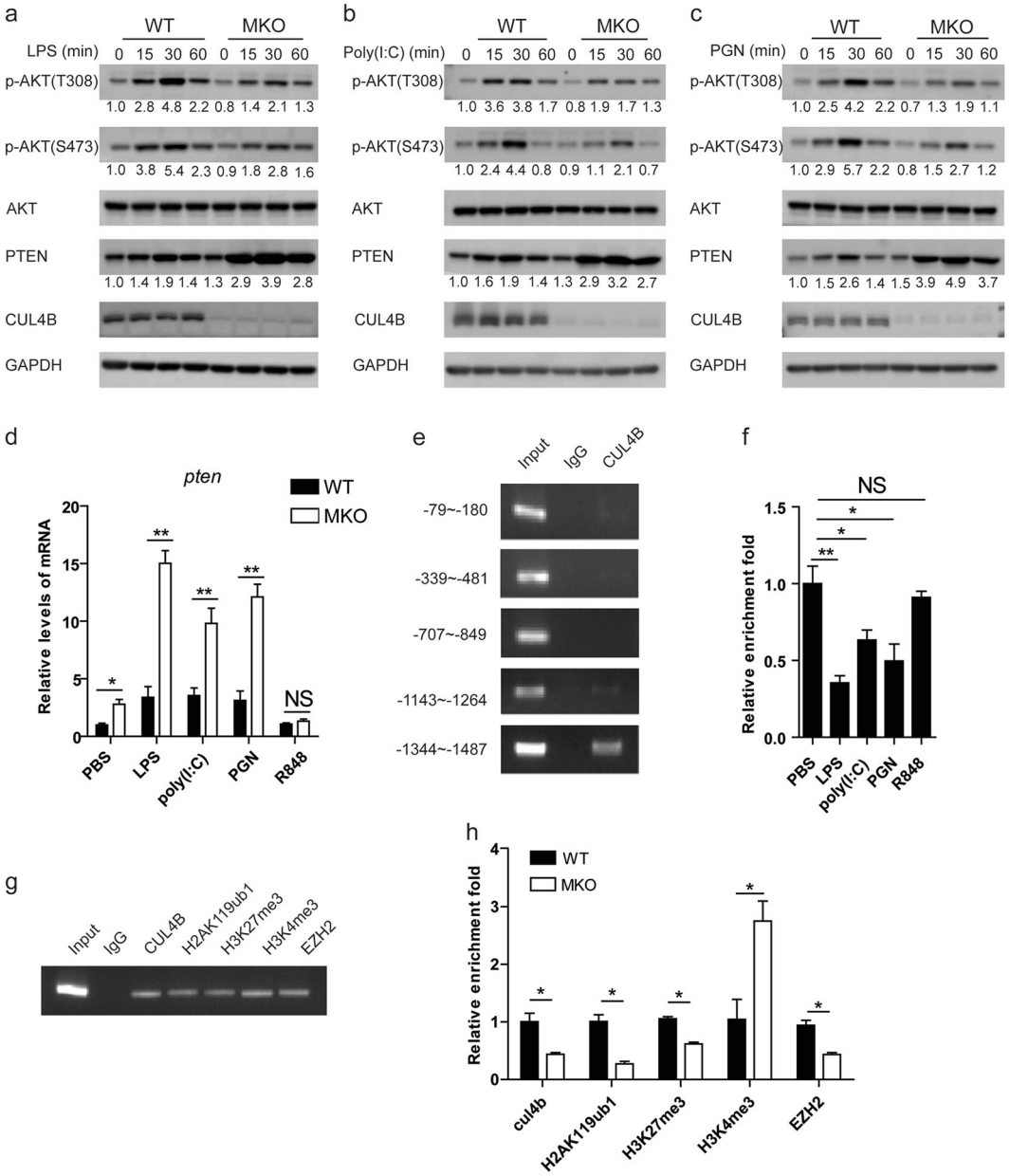


**Fig. 6** Transcriptional upregulation of *Pten* contributes to enhanced GSK3β activity in CUL4B-deficient macrophages. **a–c** Effects of CUL4B deficiency on AKT activation and PTEN levels in BMDMs. WT and MKO BMDMs were treated with LPS (100 ng/ml) (**a**), poly(I:C) (50 μg/ml) (**b**), or PGN (20 μg/ml) (**c**) for the indicated times, and cells were then harvested and lysed for immunoblot analysis with the indicated antibodies. Band intensities given underneath gel images were measured using ImageJ software and are presented as a fold change. **d** Effects of CUL4B deficiency on *Pten* transcription in BMDMs. WT and MKO BMDMs were stimulated with LPS (100 ng/ml), poly(I:C) (50 μg/ml), or PGN (20 μg/ml) for 30 min, and the mRNA levels of *Pten* were analyzed by qRT-PCR. **e** ChIP assay of CUL4B binding to the *Pten* promoter. **f** The effect of TLR agonist stimulation on CUL4B binding to the *Pten* promoter. BMDMs were stimulated with LPS, poly(I:C) or PGN for 30 min, following which qChIP assays were performed. **g** ChIP analysis of binding of the indicated proteins to the *Pten* promoter. **h** qChIP analysis of binding of the indicated proteins to the *Pten* promoter in WT and MKO BMDMs. The data in **d**, **f** and **h** are presented as the mean ± SD. Data are representative of more than three independent experiments. *$P < 0.05$; **$P < 0.01$

(Fig. 6h). Taken together, these results indicate that the CUL4B complex represses the transcription of *Pten*, which inhibits AKT and thus restrains GSK3β activity.

## DISCUSSION

Given the critical functions of TLR signaling in innate immunity, the identification and characterization of TLR signaling regulators is important for understanding how immune responses are tightly regulated. In this study, we demonstrate that CUL4B serves as a physiologic inhibitor to prevent excessive activation of TLR signaling and protect the host from being overwhelmed by inflammatory responses by restricting GSK3β activity and the subsequent production of pro-inflammatory and anti-inflammatory cytokines. We have provided several lines of evidence to support this claim. First, the lack of CUL4B in macrophages significantly augmented TLR-induced proinflammatory cytokine production and decreased IL-10 expression induced by TLRs. Second, myeloid cell-specific *Cul4b* knockout mice exhibited increased susceptibility to septic shock when challenged

with LPS, poly(I:C) or *S. typhimurium* infection. Third, regulation of the TLR-triggered immune response by CUL4B was shown to be mediated by the suppression of proinflammatory GSK3β activity. The inhibition of GSK3β efficiently blocked the increase in TLR-triggered inflammation caused by CUL4B deletion and protected mice from *S. typhimurium*-induced endotoxin shock. Finally, the CRL4B complex was shown to regulate AKT-GSK3β activity by epigenetically repressing *Pten* transcription. However, our results differ from those of a report by Hung et al.,[44] in which the authors showed that the deletion of CUL4B in macrophages enhanced LPS-induced peritonitis but not production of the proinflammatory cytokines IL-6 and TNF-α.

Proper control of GSK3β activity appears to be quite important for a variety of cellular processes, including immune responses.[14,16,45] Accumulating evidence indicates that GSK3β regulates pro-inflammatory and anti-inflammatory cytokine production in TLR signaling.[15–17,41] Martin et al first demonstrated the regulatory roles of GSK3β in diverse TLR-mediated inflammatory cytokine production.[16] TLR agonist stimulation led to the phosphorylation and inactivation of GSK3β through a PI3K/AKT-dependent pathway.[16,18,42,46] Consistent with these observations, we found that the dysregulation of pro-inflammatory and anti-inflammatory cytokine production in pathogen-triggered CUL4B-deficient macrophages was also mediated by enhanced GSK3β activity. Enhanced GSK3β activity in CUL4B-deficient BMDMs promoted the production of proinflammatory cytokines and inhibited production of the anti-inflammatory cytokine IL-10. Accordingly, we showed that the pharmacologic inhibition of GSK3β could efficiently block the enhanced TLR-triggered inflammatory response caused by CUL4B deletion in vitro and in vivo. Several mechanisms are involved in the regulation of GSK3β activity.[41,45,47] We found that decreased GSK3β phosphorylation at Ser9 is associated with decreased AKT kinase activity. Intriguingly, while CUL4B negatively regulates GSK3β activity by repressing the phosphatases PP2A and PHLPP1/2, which dephosphorylate and inactivate AKT to sustain Wnt signaling in myeloid-derived suppressive cells (MDSCs),[30] no significant difference in the levels of PP2A and PHLPP was found between *Cul4b*-deficient and control macrophages. However, PTEN, a negative regulator of AKT, was found to be significantly elevated in CUL4B-deficient macrophages. These findings suggest that AKT signaling is regulated differently in MDSCs and BMDMs. While decreased AKT activity was shown to affect other downstream targets, such as the mTOR signaling pathway and thereby NF-κB,[48,49] we detected no significant changes in these targets in CUL4B-deficient macrophages (data not shown). Importantly, the pharmacological inhibition of GSK3β activity efficiently blocked the increase in TLR-triggered proinflammatory cytokine production and attenuated TLR-triggered death caused by CUL4B deletion, indicating that GSK3β plays a major role in mediating negative regulation of the TLR-triggered inflammatory response by CUL4B.

Previous studies have shown that the CUL4B complex targets several substrates, such as cyclin E, PPARγ, PrxIII, and Jab1, for ubiquitin-dependent proteasomal degradation.[33,34,36,50] However, an examination of TLR and its downstream signaling molecules MYD88, TRAF6, TRAF3, TAK1, and TRIF revealed no difference in their levels between WT and MKO cells (data not shown). In addition to its role in promoting protein degradation, the CUL4B complex functions as a transcriptional corepressor of gene transcription.[37,43,51] We showed here that the CUL4B complex epigenetically represses the expression of *Pten*, which subsequently inhibits AKT-GSK3β signaling, subsequently increasing NF-κB activity and decreasing CREB activity. The AKT-GSK3β-CREB pathway has been shown to be critical for the regulation of IL-10 production during TLR activation.[16] Consistent with these reports, elevated NF-kB activity and attenuated CREB activation were observed in TLR-induced CUL4B-deficient macrophages. Increased proinflammatory cytokine and decreased anti-inflammatory cytokine production is presumably responsible for the aggravated inflammatory responses observed in *Cul4b*-deficient mice.

Although CUL4B expression levels were not changed during the TLR response, we observed decreased binding of CUL4B to the *Pten* promoter during TLR agonist stimulation. The CUL4B complex does not contain a DNA-binding domain and may target specific genomic sites via its partner proteins, such as transcriptional factors. However, the identity of the transcription factor that recruits the CUL4B complex to the *Pten* gene promoter remains unknown. Future studies are needed to determine how CUL4B is recruited to the *Pten* promoter and which transcription factor(s) is used to specifically target the *Pten* promoter during the TLR response.

In summary, we demonstrated that CUL4B can restrict TLR-triggered inflammatory responses by regulating GSK3β activity. Our results reveal a CRL4B-based epigenetic mechanism by which TLR-mediated inflammatory responses are modulated and may have implications for the development of novel anti-inflammatory strategies to manage infections and other diseases.

## MATERIALS AND METHODS

### Mice

*Cul4b* floxed mice were developed as previously reported.[28] To produce conditional knockout mice in which *Cul4b* was specifically deleted in myeloid cells, *Cul4b* floxed mice were crossed with LysM-Cre transgenic mice, producing $Cul4b^{flox/Y}LysM\text{-}Cre^{+/-}$ (MKO) mice and $Cul4b^{flox/Y}LysM\text{-}Cre^{-/-}$ (WT) littermate controls. Mice were housed in a specific pathogen-free animal facility at Shandong University. Animal care and experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Shandong University School of Basic Medical Sciences.

### Generation of bone marrow–derived macrophages and peritoneal macrophages

Bone marrow–derived macrophages (BMDMs) were generated as previously described.[52] Bone marrow cells ($1 \times 10^7$) were cultivated in RPMI 1640 medium containing 10% FBS and 10 ng/ml recombinant murine M-CSF (PeproTech) in a 100-mm dish for 5 days to generate BMDMs.

Peritoneal macrophages (PMs) were obtained from thioglycollate-elicited mice and rinsed with ice-cold PBS. Cells were resuspended in the relevant medium (DMEM supplemented with 10% FBS, 2 g/l sodium bicarbonate, 100 mg/l sodium pyruvate, 10 mM HEPES (pH 7.4), 62.1 mg/l penicillin, and 100 mg/l streptomycin), seeded in plates with a density of $10^6$/ml, and subsequently cultured overnight at 37 °C. Nonadherent cells were discarded by rinsing with PBS.

### In vivo endotoxic shock model

LPS (10 μg/g; Sigma-Aldrich) was intraperitoneally injected into age-matched and sex-matched WT and MKO mice to induce LPS shock. *S. typhimurium* strain SL1344 was diluted in PBS and administered to WT ($5 \times 10^4$ CFU/ml) or MKO ($5 \times 10^4$ CFU/ml) mice through tail vein injection. Colony-forming units (CFUs) were measured by counting viable bacteria on agar plates in blood samples. For poly(I:C) induction, age-matched and sex-matched WT mice and MKO mice were i.p. injected with poly(I:C) (5 μg/g body weight; ApexBio) and D-galactosamine (0.5 mg/g body weight; Sigma-Aldrich). Mouse survival was monitored, and serum and target organs were collected at the specified times.

### Cell culture and cytokine assay

BMDMs were produced with recombinant murine M-CSF (50 ng/ml). After their incubation for 6 days, the cells were stimulated with LPS (100 ng/ml) or poly(I:C) (50 μg/ml). TNF-α, IL-6,

IL-1β, IL-10, and IFN-β in supernatants and sera were quantified by sandwich ELISA (BioLegend) following the manufacturer's instructions.

Real-time PCR
TRIzol reagent was applied to lyse the cells, and total RNA was collected according to the manufacturer's instructions (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed on a Roche 480 system. The results are expressed as the relative abundance of the specific mRNA normalized to that of Gapdh.

Western blot assays
Cells were lysed with SDS lysis buffer, lysates were obtained by centrifugation, and protein concentrations were detected with a BCA Protein Assay Kit (Thermo Scientific). Equivalent amounts of total proteins were separated by SDS-PAGE and subsequently transferred onto PVDF membranes. Proteins were detected with antibodies against specific proteins; anti-CUL4B antibody was purchased from Sigma-Aldrich, anti-PHLPP antibody was purchased from Proteintech Group, and antibodies against the rest of the proteins were purchased from CST.

Transcription factor activity assays
To prepare nuclear extracts, BMDMs from a single mouse were washed with precooled phosphatase inhibitor buffer (12.5 mM β-glycerophosphate, 6.25 mM NaF, 12.5 mM PNPP, 1.25 mM $Na_3VO_4$ in PBS) and centrifuged at $100 \times g$ and 4 °C for 6 min. Nuclear extracts were prepared using Cayman's nuclear extraction kit according to the manufacturer's instructions. Activation of NF-κB and CREB was quantified using NF-κB and pCREB/CREB assay kits (Cayman), respectively, according to the manufacturer's instructions.

Flow cytometric analysis
Single-cell suspensions were freshly prepared from mouse bone marrow and spleens. Cells were stained with anti-CD11b (BioLegend), anti-Gr-l (eBioscience), anti-Ly6C (BioLegend), and anti-F4/80 (BioLegend) antibodies. Data were analyzed with FlowJo 7.6.5 software.

Chromatin immunoprecipitation analysis
ChIP was performed as described previously.[37] Briefly, $1 \times 10^7$ cells were crosslinked with 1% formaldehyde, sonicated, precleared and incubated with 5–10 mg of antibody per reaction. The complexes were rinsed with low-salt and high-salt buffers, and the relevant DNA was extracted and precipitated. Primer sequences are listed in Supplementary Tables S1.

Statistical analysis
The data are reported as the mean ± SEM from the indicated number of mice or biologic replicates and as the mean ± SD of the indicated number of technical replicates. Statistical analysis was performed with GraphPad Prism 5.0 software. Differences between means were analyzed by unpaired Student's *t*-test. The statistical significance of survival differences was estimated with the Kaplan-Meier method, and curves were compared with the generalized Wilcoxon test. *P* values of less than 0.05 indicated statistical significance.

## ACKNOWLEDGEMENTS
We thank Prof. Chengjiang Gao and Prof. Wei Zhao for their critically reading of the paper. This work was supported by the National Natural Science Foundation of China (81571523 and 31872810), the Natural Science Foundation of Shandong Province (ZR2016HZ01), and the Key Research and Development Program of Shandong Province (2016ZDJS07A08 and 2017GSF218027).

## ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41423-019-0323-0) contains supplementary material.

**Competing interests:** The authors declare no competing interests.

## REFERENCES
1. Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**, 675–680 (2001).
2. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat. Rev. Immunol.* **4**, 499–511 (2004).
3. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805–820 (2010).
4. Savva, A. & Roger, T. Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. *Front. Immunol.* **4**, 387 (2013).
5. Broz, P. & Monack, D. M. Newly described pattern recognition receptors team up against intracellular pathogens. *Nat. Rev. Immunol.* **13**, 551–565 (2013).
6. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637–650 (2011).
7. O'Neill, L. A. & Bowie, A. G. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* **7**, 353–364 (2007).
8. Seki, E. & Brenner, D. A. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* **48**, 322–335 (2008).
9. Dugo, L. et al. Insulin reduces the multiple organ injury and dysfunction caused by coadministration of lipopolysaccharide and peptidoglycan independently of blood glucose: role of glycogen synthase kinase-3beta inhibition. *Crit. Care Med.* **34**, 1489–1496 (2006).
10. Jope, R. S., Yuskaitis, C. J. & Beurel, E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem. Res.* **32**, 577–595 (2007).
11. Rayasam, G. V., Tulasi, V. K., Sodhi, R., Davis, J. A. & Ray, A. Glycogen synthase kinase 3: more than a namesake. *Br. J. Pharm.* **156**, 885–898 (2009).
12. Beurel, E., Michalek, S. M. & Jope, R. S. Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3). *Trends Immunol.* **31**, 24–31 (2010).
13. Woodgett, J. R. Judging a protein by more than its name: GSK-3. *Sci. STKE* **2001**, re12 (2001).
14. Wang, H., Brown, J. & Martin, M. Glycogen synthase kinase 3: a point of convergence for the host inflammatory response. *Cytokine* **53**, 130–140 (2011).
15. Ko, R., Park, J. H., Ha, H., Choi, Y. & Lee, S. Y. Glycogen synthase kinase 3beta ubiquitination by TRAF6 regulates TLR3-mediated pro-inflammatory cytokine production. *Nat. Commun.* **6**, 6765 (2015).
16. Martin, M., Rehani, K., Jope, R. S. & Michalek, S. M. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* **6**, 777–784 (2005).
17. Hu, X. et al. IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. *Immunity* **24**, 563–574 (2006).
18. Zhang, Y. et al. Activation of vascular endothelial growth factor receptor-3 in macrophages restrains TLR4-NF-kappaB signaling and protects against endotoxin shock. *Immunity* **40**, 501–514 (2014).
19. Jackson, S. & Xiong, Y. CRL4s: the CUL4-RING E3 ubiquitin ligases. *Trends Biochem. Sci.* **34**, 562–570 (2009).
20. Sarikas, A., Hartmann, T. & Pan, Z. Q. The cullin protein family. *Genome Biol.* **12**, 220 (2011).
21. Hannah, J. & Zhou, P. Distinct and overlapping functions of the cullin E3 ligase scaffolding proteins CUL4A and CUL4B. *Gene* **573**, 33–45 (2015).
22. Tarpey, P. S. et al. Mutations in CUL4B, which encodes a ubiquitin E3 ligase subunit, cause an X-linked mental retardation syndrome associated with aggressive outbursts, seizures, relative macrocephaly, central obesity, hypogonadism, pes cavus, and tremor. *Am. J. Hum. Genet.* **80**, 345–352 (2007).
23. Zou, Y. et al. Mutation in CUL4B, which encodes a member of cullin-RING ubiquitin ligase complex, causes X-linked mental retardation. *Am. J. Hum. Genet.* **80**, 561–566 (2007).
24. Kerzendorfer, C. et al. CUL4B-deficiency in humans: understanding the clinical consequences of impaired Cullin 4-RING E3 ubiquitin ligase function. *Mech. Ageing Dev.* **132**, 366–373 (2011).
25. Badura-Stronka, M. et al. A novel nonsense mutation in CUL4B gene in three brothers with X-linked mental retardation syndrome. *Clin. Genet.* **77**, 141–144 (2010).
26. Isidor, B., Pichon, O., Baron, S., David, A. & Le Caignec, C. Deletion of the CUL4B gene in a boy with mental retardation, minor facial anomalies, short stature, hypogonadism, and ataxia. *Am. J. Med. Genet. A* **152a**, 175–180 (2010).

27. Lee, J. & Zhou, P. Pathogenic role of the CRL4 ubiquitin ligase in human disease. *Front Oncol.* **2**, 21 (2012).
28. Jiang, B. et al. Lack of Cul4b, an E3 ubiquitin ligase component, leads to embryonic lethality and abnormal placental development. *PLoS ONE* **7**, e37070 (2012).
29. Liu, L. et al. Essential role of the CUL4B ubiquitin ligase in extra-embryonic tissue development during mouse embryogenesis. *Cell Res.* **22**, 1258–1269 (2012).
30. Qian, Y. et al. The CUL4B/AKT/beta-catenin axis restricts the accumulation of myeloid-derived suppressor cells to prohibit the establishment of a tumor-permissive microenvironment. *Cancer Res.* **75**, 5070–5083 (2015).
31. Zhao, W. et al. Lack of CUL4B leads to increased abundance of GFAP-positive cells that is mediated by PTGDS in mouse brain. *Hum. Mol. Genet*. **24**, 4686–4697 (2015).
32. Zhao, Y. & Sun, Y. CUL4B ubiquitin ligase in mouse development: a model for human X-linked mental retardation syndrome? *Cell Res.* **22**, 1224–1226 (2012).
33. Li, P. et al. Lack of CUL4B in adipocytes promotes PPARgamma-mediated adipose tissue expansion and insulin sensitivity. *Diabetes* **66**, 300–313 (2017).
34. Li, X. et al. Cullin 4B protein ubiquitin ligase targets peroxiredoxin III for degradation. *J. Biol. Chem.* **286**, 32344–32354 (2011).
35. Wei, Z. et al. CUL4B impedes stress-induced cellular senescence by dampening a p53-reactive oxygen species positive feedback loop. *Free Radic. Biol. Med.* **79**, 1–13 (2015).
36. He, F. et al. X-linked intellectual disability gene CUL4B targets Jab1/CSN5 for degradation and regulates bone morphogenetic protein signaling. *Biochim. Biophys. Acta* **1832**, 595–605 (2013).
37. Hu, H. et al. CRL4B catalyzes H2AK119 monoubiquitination and coordinates with PRC2 to promote tumorigenesis. *Cancer Cell* **22**, 781–795 (2012).
38. Xia, M. et al. Histone methyltransferase Ash1l suppresses interleukin-6 production and inflammatory autoimmune diseases by inducing the ubiquitin-editing enzyme A20. *Immunity* **39**, 470–481 (2013).
39. Han, C. et al. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat. Immunol.* **11**, 734–742 (2010).
40. Yuan, J. et al. CUL4B activates Wnt/beta-catenin signalling in hepatocellular carcinoma by repressing Wnt antagonists. *J. Pathol.* **235**, 784–795 (2015).
41. Ko, R. & Lee, S. Y. Glycogen synthase kinase 3beta in Toll-like receptor signaling. *BMB Rep.* **49**, 305–310 (2016).
42. Zhang, P., Katz, J. & Michalek, S. M. Glycogen synthase kinase-3beta (GSK3beta) inhibition suppresses the inflammatory response to Francisella infection and protects against tularemia in mice. *Mol. Immunol.* **46**, 677–687 (2009).
43. Ji, Q. et al. CRL4B interacts with and coordinates the SIN3A-HDAC complex to repress CDKN1A and drive cell cycle progression. *J. Cell Sci.* **127**, 4679–4691 (2014).
44. Hung, M. H., Jian, Y. R., Tsao, C. C., Lin, S. W. & Chuang, Y. H. Enhanced LPS-induced peritonitis in mice deficiency of cullin 4B in macrophages. *Genes Immun.* **15**, 404–412 (2014).
45. Wang, H., Kumar, A., Lamont, R. J. & Scott, D. A. GSK3beta and the control of infectious bacterial diseases. *Trends Microbiol*. **22**, 208–217 (2014).
46. Wang, H. et al. The role of JAK-3 in regulating TLR-mediated inflammatory cytokine production in innate immune cells. *J. Immunol.* **191**, 1164–1174 (2013).
47. Mulholland, D. J., Dedhar, S., Wu, H. & Nelson, C. C. PTEN and GSK3beta: key regulators of progression to androgen-independent prostate cancer. *Oncogene* **25**, 329–337 (2006).
48. Brown, J., Wang, H., Hajishengallis, G. N. & Martin, M. TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *J. Dent. Res.* **90**, 417–427 (2011).
49. Weichhart, T. et al. The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* **29**, 565–577 (2008).
50. Zou, Y. et al. Characterization of nuclear localization signal in the N terminus of CUL4B and its essential role in cyclin E degradation and cell cycle progression. *J. Biol. Chem.* **284**, 33320–33332 (2009).
51. Xu, Z. et al. Upregulation of IL-6 in CUL4B-deficient myeloid-derived suppressive cells increases the aggressiveness of cancer cells. *Oncogene* **38**, 5860–5872 (2019).
52. Sun, Q. et al. The specific and essential role of MAVS in antiviral innate immune responses. *Immunity* **24**, 633–642 (2006).