

E3 ubiquitin ligase CHIP facilitates Toll-like receptor signaling by recruiting and polyubiquitinating Src and atypical PKC ζ

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The carboxyl terminus of constitutive heat shock cognate 70 (HSC70)-interacting protein (CHIP, also known as Stub1) is a U box-containing E3 ubiquitin ligase that is important for protein quality control. The role of CHIP in innate immunity is not known. Here, we report that CHIP knockdown inhibits Toll-like receptor (TLR) 4- and TLR9-driven signaling, but not TLR3-driven signaling; proinflammatory cytokine and type 1 interferon (IFN) production; and maturation of antigen-presenting cells, including macrophages and dendritic cells. We demonstrate that CHIP can recruit the tyrosine kinase Src and atypical protein kinase C ζ (PKC ζ) to the TLR complex, thereby leading to activation of IL-1 receptor-associated kinase 1, TANK-binding kinase 1, and IFN regulatory factors 3 and 7. CHIP acts as an E3 ligase for Src and PKC ζ during TLR signaling. CHIP-mediated enhancement of TLR signaling is inhibited by IFNAR deficiency or expression of ubiquitination resistant mutant forms of Src or PKC ζ . These findings suggest that CHIP facilitates the formation of a TLR signaling complex by recruiting, ubiquitinating, and activating Src and PKC ζ .

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Abbreviations used: BMDC, BM-derived DC; CHIP, carboxyl terminus of constitutive HSC70-interacting protein; CpG ODN, CpG oligodeoxynucleotides; HEK, human embryonic kidney; HSC70, heat shock cognate 70; HSP70, heat shock protein 70; IRAK, IL-1 receptor-associated kinase; IRF, IFN regulatory factor; MyD88, myeloid differentiation factor 88; PKC, protein kinase C; RNAi, RNA interference vector; siRNA, small interfering RNA; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, Toll/IL-1R domain-containing adapter-inducing IFN- β .

Toll-like receptors (TLRs) play important roles in both innate and adaptive immunity (Kawai and Akira, 2010; McGettrick and O'Neill, 2010). By recognizing structurally conserved pathogen components termed pathogen-associated molecular patterns, TLRs activate signaling through the Toll/IL-1R (TIR) domain, which in turn triggers the binding of the TIR domain-containing adaptors, including myeloid differentiation factor 88 (MyD88), TIRAP (Mal), Toll/IL-1R domain-containing adapter-inducing IFN- β (TRIF), and TRAM and activates specific signaling pathways (Kawai and Akira, 2010; McGettrick and O'Neill, 2010). MyD88 activates the IL-1 receptor-associated kinases (IRAKs) IRAK4 and IRAK1, which finally activates IKK-NF- κ B and mitogen-activated protein kinases (MAPK; Medzhitov et al., 1998). TRIF recruits tumor necrosis factor receptor-associated factor 6 (TRAF6) and activates transforming growth factor-activated kinase-1 (TAK1) for NF- κ B activation (Jiang et al., 2004). IFN regulatory factor (IRF) 3 activation by TRIF requires the activation of TANK-binding

kinase I (TBK1)/IKK ϵ , leading to the production of type I IFN (IFN- α/β ; Fitzgerald et al., 2003), which is important for innate elimination of virus and adaptive induction of CD8⁺ T cells (Gautier et al., 2005; Mattei et al., 2009). Different from the TRIF-induced activation of IRF3, the MyD88-dependent pathway used by TLR2, 5, 7, and 9 can induce type I IFN production by MyD88-dependent activation of IRF5 or IRF7 (Kawai and Akira, 2010; McGettrick and O'Neill, 2010). Macrophages and DCs are professional APCs that are involved in both innate immunity and adaptive immunity, mainly by producing cytokines upon encountering various pathogens. Despite the fact that the TLR signaling pathway has been outlined, more efforts are required for elucidation of molecules involved in the regulation of TLR signaling (e.g., assembly of TLR proximal

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complex and activation of serine–threonine kinases), and, more importantly, the regulation of APC functions by TLR-mediated signaling.

The process of protein ubiquitination, which is mediated by enzymes known as E1, E2, and E3, and the process of protein deubiquitination, which is mediated by deubiquitylating enzymes (DUBs), play important roles in the modulation of immune responses (Liu et al., 2005). For TLR signaling, effects and mechanisms of several E3 ligases and DUBs have been reported (Liu et al., 2005). TRAF6 synthesizes a K63-linked polyubiquitin chain on target proteins, including NEMO and TRAF6 itself, leading to activation of TAK1 and NEMO (Deng et al., 2000). In the TRIF-dependent TLR3/4 signaling pathway, TRAF3 is essential for the K63-linked ubiquitination of TANK (Gatot et al., 2007). In contrast to K63-linked polyubiquitination and activation, the K48-linked polyubiquitination can target the substrates to proteasome degradation, restricting the innate immune response (Liu et al., 2005). Several E3s have been implicated in the negative regulation of TLR signaling, such as Triad3A, SOCS1, Cbl-b, PDLIM2, A20, and Itch (Liu et al., 2005; Kawai and Akira, 2010; McGettrick and O'Neill, 2010). Therefore, the manner of ubiquitination by E3 and the contact of ubiquitinated substrates with DUBs may decide the intensity and the fate of TLR signaling components. Many efforts have been put into the study of E3-mediated regulation of T cell signaling (Liu et al., 2005). However, the functional regulation of APCs, which are the bridge between innate immunity and adaptive immunity, by E3s and underlying mechanisms has not been completely elucidated (Gautier et al., 2005; Liu et al., 2005; Mattei et al., 2009; Kawai and Akira, 2010; McGettrick and O'Neill, 2010).

We have previously demonstrated that the E3 ligase Nrdp1 can modulate the ubiquitination of MyD88 and TBK1, leading to preferential IFN- β production (Wang et al., 2009). Type I IFN produced by APCs such as DCs and macrophages, plays an important role in regulating the cytokine production, survival, maturation, and antigen presentation of APCs by activating IFN- α/β receptor (IFNAR)-mediated transcriptional regulation of type I IFN-induced genes (Gautier et al., 2005; Mattei et al., 2009; Kawai and Akira, 2010; McGettrick and O'Neill, 2010). To elucidate the mechanisms involved in the functional regulation of APCs, we screened and investigated the effects of E3s in regulating TLR-mediated cytokine production by using small interfering RNAs (siRNAs) for E3s in DCs. We found that knockdown of one of the E3s, the carboxyl terminus of constitutive heat shock cognate 70 (HSC70)-interacting protein (CHIP; also known as Stub1), significantly inhibited LPS (for TLR4) and unmethylated CpG oligodeoxynucleotides (CpG ODN or CPG; for TLR9)-induced production of IFN- β . Previous studies suggest that CHIP contains a ring finger-like U box domain and acts as E3 in combination with chaperones heat shock protein 70 (HSP70) and HSP90 to mediate the ubiquitination of chaperone-bound substrates (Ballinger et al., 1999; Connell et al., 2001; Dickey et al., 2007). CHIP has been reported to

mediate the ubiquitination of diverse proteins, including membrane receptors, transcription factors, kinases, and several pathological proteins (e.g., Ballinger et al., 1999; Connell et al., 2001; Dickey et al., 2007). Knockout of CHIP in mice leads to atrophy and temperature-sensitive apoptosis in multiple organs, impairs heat stress responses and protein folding, decreases longevity, and accelerates aging (Dai et al., 2003; Morishima et al., 2008; Maruyama et al., 2010; Naito et al., 2010). However, the roles of CHIP in immunity have not been elucidated. In the current study, we investigate the effect of CHIP on the regulation of TLR signaling in macrophages and DCs.

RESULTS

CHIP knockdown inhibits TLR-triggered innate response

We performed a siRNA-based screening of potential E3s in regulating APC functions. In BM-derived DCs (BMDCs), we examined the effects of knockdown of several E3s (such as Cbl-b, GRAIL, A20, SOCS1, TRAF6, ITCH, MARCH-I, AIRE, CHIP, etc.) on TLR4-triggered production of TNF and IFN- β (Fig. S1, A–D). Additionally, we showed that IFN- β production was not caused by the siRNA duplexes used in this study (10 or 30 nM; Fig. S1 E). Knockdown of one of the E3s (Fig. S2, A and B), CHIP, could significantly inhibit TNF and IFN- β production by DCs after stimulation with LPS (Fig. S2, C and D). Overexpression of a same-sense CHIP mutant resistant to siRNA4 (CHIP-R4) could rescue CHIP silence-induced inhibition of TNF and IFN- β production (Fig. S2, C and D).

CHIP is widely expressed in immune cells, including peritoneal macrophages, RAW264.7 cells, CD11c⁺ splenic DC, GM-CSF-differentiated BMDCs (CD11c⁺), Flt-3-differentiated plasmacytoid DCs (pDCs; CD11c⁺B220⁺), DX5⁺ NK cells, CD4⁺/CD8⁺ T cells, and CD20⁺ B cells (Fig. S3 A). As the E3 that contains U box, the role of CHIP in immunity has never been elucidated. Therefore, we investigated the effects and the underlying mechanisms of CHIP-mediated regulation of TLR signaling in macrophages and DCs. In macrophages, knockdown of CHIP by siRNA4 (Fig. S3 B) could significantly inhibit IL-6 and IFN- β production triggered by TLR2, TLR4, TLR7, and TLR9, but not TLR3, signaling (Fig. 1). CHIP knockdown could also inhibit the production of TNF induced by TLR2/4/7/9 signaling at an early stage (1 h after LPS treatments) in macrophages (Fig. S3 C). In BMDCs and pDCs, CHIP knockdown (Fig. S3 D) inhibited TLR2/4/7/9 agonist-induced IL-6 and IFN- β production (Fig. 1). Moreover, CHIP knockdown inhibited TLR2/4/9-induced IL-12p70 production (Fig. S4 A) and maturation of BMDCs (Fig. S4 B). These results suggest that CHIP may be required for TLR2/4/7/9-induced activation of macrophages and DCs.

Previously, it has been suggested that extracellular poly(I:C) can activate both TLR3 and MDA-5. To confirm whether CHIP was involved in TLR3 signaling, we examined the effects of CHIP silence in poly(I:C)-triggered IRF3 reporter activation in TLR3-transfected MDA-5^{low} human embryonic

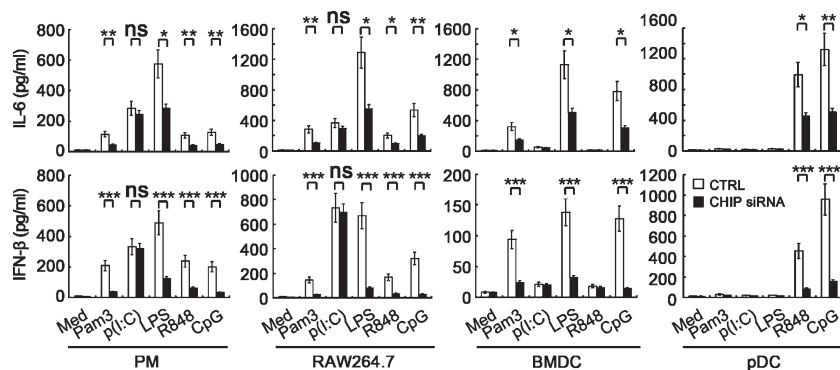


Figure 1. Silencing of CHIP impairs TLR-triggered innate response in macrophages and DCs.

48 h after transient transfection of 30-nM siRNAs in indicated cells, the cells were treated with 160 ng/ml Pam3Cys (Pam3), 10 μg/ml poly (I:C) (p(I:C)), 100 ng/ml LPS, 2 μg/ml R848, or 1 μM CpG ODN for 4 h (for macrophages) or 8 h (for DCs). Results were presented as mean concentration (picogram/milliliter) of indicated cytokines in the culture supernatants ± SD of triplicate samples. CTRL, scrambled control siRNAs; PM, peritoneal macrophages; BMDCs, BM-derived DCs; pDCs, plasmacytoid DCs; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data shown correspond to a representative experiment out of three performed.

kidney 293 (HEK293) cells and in MDA5^{-/-} MEFs. We found that poly(I:C) treatments could activate IRF3 reporter in both cells while CHIP silencing didn't affect the effects of poly(I:C) (Fig. S5, A–C), indicating that CHIP may be not required for TLR3 signaling.

CHIP knockdown inhibits NF-κB and IRF3/7

To look into the potential signaling pathways regulated by CHIP, we performed reporter assays in RAW264.7 cells that were stably transfected with CHIP RNA interference vector (RNAi; Fig. 2 A). We found that CHIP knockdown inhibited LPS/CpG-induced activation of NF-κB and IRF3 reporters (Fig. 2 B), whereas the AP1 reporter activation by LPS/CpG was not affected (unpublished data). Correspondingly,

we found that the activation of IL-6, CCL5, and IFN-β reporters were also significantly impaired in RAW264.7 cells after CHIP knockdown (Fig. 2 C), indicating that CHIP knockdown-induced decrease of IL-6 and IFN-β production (Fig. 1) may be caused by decreased NF-κB and IRF3 activation. We also found that CHIP over-expression in RAW264.7 cells led to increased IL-6 and IFN-β production and NF-κB and IRF3 reporter activation (unpublished data).

To further examine the effects of CHIP knockdown on NF-κB and IRF3/7 activation, we examined the DNA-binding capacity and nuclear translocation of NF-κB and the nuclear translocation of IRF3/7. In CHIP-silenced RAW264.7 cells, the nuclear presence of p50, p65, IRF3, and IRF7 after LPS/CpG treatments was decreased (Fig. 3 A). Moreover, the DNA-bound NF-κB levels and nuclear IRF3/7 levels were reduced in CHIP-silenced RAW264.7 cells (Fig. 3, B and C). In BMDCs, CHIP knockdown inhibited TLR4/9-triggered NF-κB activation (Fig. 3 D) and nuclear translocation of IRF3 and IRF7 (Fig. 3 E). Similarly, CHIP knockdown inhibited TLR9-triggered NF-κB activation (Fig. 3 D) and nuclear translocation of IRF3 and IRF7 in pDCs (Fig. 3 E).

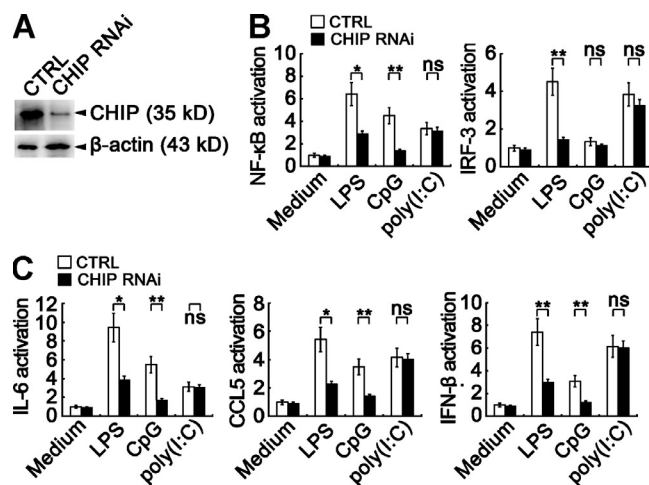


Figure 2. Silencing of CHIP impairs TLR signaling pathways.

(A) RAW264.7 cells were transfected with plasmids encoding siRNAs, and selected with 600 μg/ml neomycin. The efficiency of silencing was evaluated by Western blot. CTRL, scrambled control RNAi; RNAi, CHIP-specific RNAi. (B and C) Reporter assays in RAW264.7 cells. Cells in A were transiently transfected with NF-κB or IRF3 reporters (B) or IL-6, CCL5, or IFN-β promoter reporters for 48 h, and then treated with 100 ng/ml LPS, 10 μg/ml poly (I:C), or 1 μM CpG ODN for 4 h. Results were expressed as fold induction relative to the luciferase activity in unstimulated cells transfected with control vector. Data are shown as mean ± SD of triplicate samples. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. The data shown correspond to a representative experiment out of three performed.

CHIP interacts with Src, protein kinase C ζ (PKCζ), and TRAF6

CHIP was initially identified as an E3 interacting with HSP70 and HSP90 (Ballinger et al., 1999; Connell et al., 2001; Dickey et al., 2007). However, its molecular mechanisms involved in TLR response have not been examined. In LPS/CpG-treated RAW264.7 cells, we found that CHIP, together with MyD88, TRIF, IRAK1, TBK1, TRAF6, HSC70, Src, or PKCζ, could be coimmunoprecipitated with TLR4 and TLR9 (but not TLR3; Fig. 4 A), indicating that CHIP may be a component of a TLR4/9 complex that initiates signaling.

Next, we investigated the CHIP-associated molecules by using recombinant GST fusion proteins (Fig. 4 B, top). By using cell lysates from RAW264.7 cells, we found that CHIP can associate with HSC70, PKCζ, Src, and TRAF6 (Fig. 4 B, middle), but not MyD88, TRIF, IRAK1, and TBK1 (not depicted), which suggests the possible association of CHIP with HSC70, PKCζ, Src, and TRAF6. To verify whether CHIP can directly interact with these targets, we performed

GST pull-down assays using recombinant proteins, and found that CHIP could directly bind HSC70/PKCζ/Src via TPR domain (Fig. 4 B, bottom).

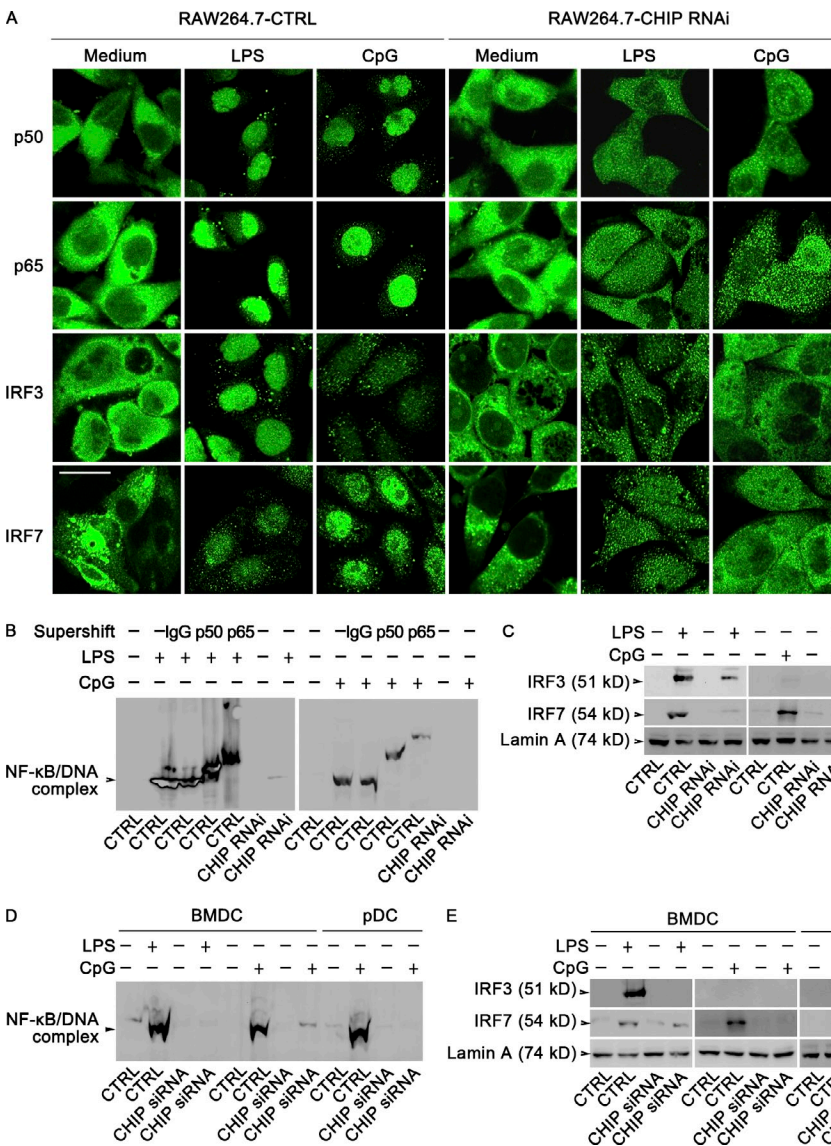
To further verify these interactions, we coexpressed HA-tagged CHIP with Flag-tagged HSC70, PKCζ, Src, or TRAF6 in HEK293 cells and found that CHIP can associate with these Flag-tagged molecules (Fig. 4 C) and may associate with TRAF6 indirectly via HSC70 (Fig. 4, B and C).

CHIP recruits and activates Src and PKCζ

Next, we investigated the effects of CHIP in TLR signaling components. In CHIP-silenced RAW264.7 cells, LPS/CpG-induced recruitment of Src and PKCζ by TLR4/9 was decreased, whereas the recruitment of HSC70 and TRAF6 by TLR4/9 was not affected (Fig. 5 A). The recruitment of MyD88, TRIF, IRAK1, and TBK1 by TLR4/9 was not affected by CHIP knockdown (unpublished data). Consistent with the observations that CHIP knockdown didn't affect

poly(I:C)-triggered activation of NF-κB and IRF3-IFN-β reporters (Fig. 2, B and C), we found that CHIP knockdown didn't affect the recruitment of Src to TLR3 complex (Fig. S5 D).

Accordingly, we found that LPS/CpG-induced activation of Src and PKCζ was inhibited by CHIP knockdown, as indicated by decreased tyrosine phosphorylation of Src, decreased threonine phosphorylation of PKCζ (Fig. 5 B), and decreased kinase activities of endogenous Src and PKCζ (Fig. S6 A). In RAW264.7 cells stably transfected with CHIP-HA, LPS/CpG-induced activation of Src and PKCζ was enhanced (Fig. 5 C and Fig. S6 B). Furthermore, we found that Src inhibitor PP1 could decrease the phosphorylation and kinase activity of PKCζ, and the pseudosubstrate of PKCζ (PS) could decrease the phosphorylation and kinase activity of Src (Fig. 5 C and Fig. S6 B), indicating that an interplay may exist between the recruited Src and PKCζ. These data suggest that CHIP recruits and activates Src and PKCζ in TLR response.



CHIP promotes TLR signaling via Src and PKCζ

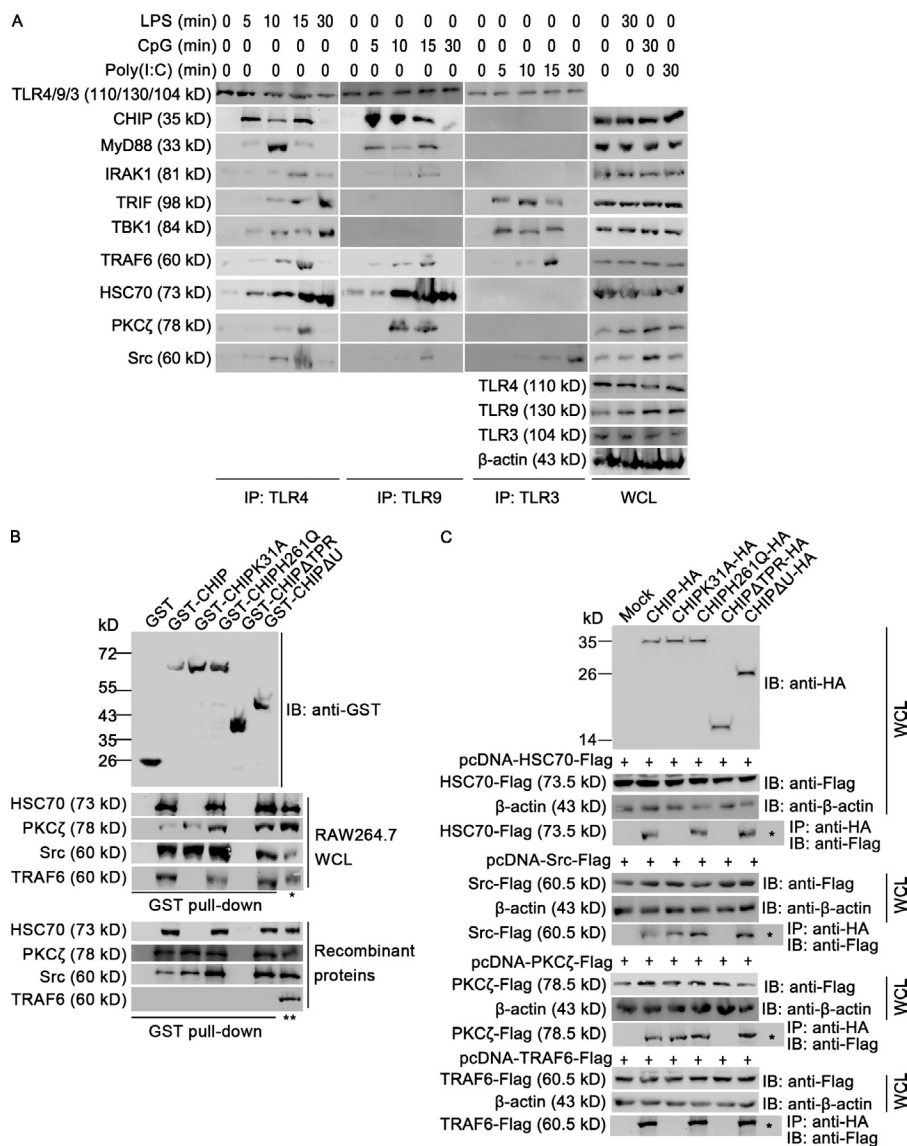
We have shown that CHIP knockdown inhibited TLR4/9-induced activation of NF-κB and IRF3/7 (Fig. 2 and Fig. 3). However, the mechanisms responsible for CHIP-mediated effects in TLR response have not been elucidated. Thus, we examined the activation status of IKKα/β and IκBα molecules, as well as TBK1 and IRAK1,

Figure 3. CHIP knockdown inhibits NF-κB and IRF3/7.

(A) RAW264.7 cells stably transfected with scrambled control vector (RAW264.7-CTRL) or CHIP silencing vector (RAW264.7-CHIP RNAi) were treated with 100 ng/ml LPS or 1 μM CpG ODN for 1 h. Next, the intracellular translocation of p50, p65, IRF3, and IRF7 were evaluated by confocal microscopy using primary antibodies (Ab) specific for the indicated proteins and Oregon Green 488-conjugated secondary antibody. Bar, 150 μm. (B and C) Cells in A were lysed and nuclear extracts were isolated. Next, DNA-binding capacity of NF-κB was examined by EMSA (B), and the nuclear translocation of IRF3/7 was examined by Western blot (C). For supershift assays in B, 1 μg of control IgG, anti-p50, or anti-p65 antibodies, where indicated, were incubated for 30 min on ice before the adding of probes. (D and E) 48 h after transient transfection of siRNAs in day 6 BMDCs and day 10 pDCs, the cells were treated with indicated TLR agonists for 1 h, and nuclear extracts were used in EMSA assays (D) or examined for nuclear IRF3/7 by Western blot (E). The data shown correspond to a representative experiment out of three performed.

molecules upstream of NF- κ B and IRF3/7, respectively. We found that in CHIP-overexpressed RAW264.7 cells and CHIP-silenced RAW264.7 cells LPS/CpG-induced phosphorylation of ERK1/2 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), p38 (Thr180/Tyr182), and IKK α / β (Ser176/180) were not significantly affected by CHIP expression levels (unpublished data). Although the LPS/CpG-induced phosphorylation of I κ B α (Ser32/36) was not affected, CHIP overexpression promoted LPS/CpG-induced degradation of I κ B α (Fig. 6 A) and kinase activity of IRAK1 and TBK1 (Fig. 6 B). More importantly, the Src inhibitor PP1 and PKC ζ pseudosubstrate could block these CHIP-mediated effects (Fig. 6, A and B). In CHIP-silenced RAW264.7 cells, the LPS/CpG-induced increase in kinase activity of IRAK1 and TBK1 was impaired (Fig. 6 C). These data suggest that CHIP may be required for the activation of I κ B α , IRAK1, and TBK1 through activation of Src/PKC ζ .

As demonstrated in Figs. 1–3, CHIP knockdown inhibited TLR-triggered activation of IRF3/7-IFN- β more significantly than TLR4/9-triggered activation of NF- κ B. Because CHIP promoted degradation of I κ B α (Fig. 6 A), which may explain CHIP-potentiated NF- κ B activation, we decided to examine the effects of IRAK1 and TBK1 activation on IRF3/7 by using recombinant IRF3 and IRF7 as substrates in the in vitro kinase assays. We found that IRAK1 and TBK1 immunoprecipitated from CHIP-overexpressed RAW264.7 cells could potentially increase the levels of phosphorylated IRF3 and IRF7 in vitro, which could be attenuated by transient transfection of SrcY416F and PKC ζ T410A (Fig. 6, D and E). Therefore, a CHIP-mediated increase in IL-6 and IFN- β production during TLR response may be caused by Src/PKC ζ -dependent activation of I κ B α and IRAK1/TBK1, respectively.

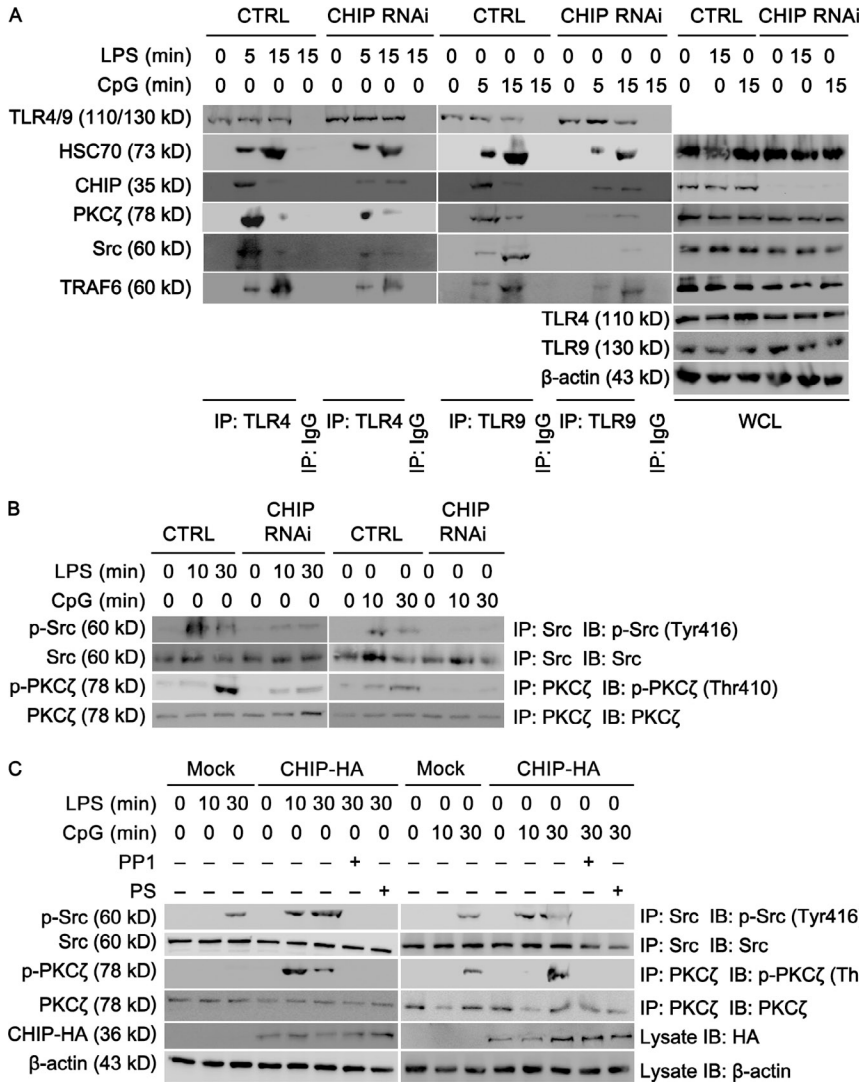


Knockdown of Src and PKC ζ blocks the effects of CHIP in TLR response

To further explore the roles of Src and PKC ζ in CHIP-mediated effects during TLR response, we silenced the expression of Src and PKC ζ with siRNAs in CHIP-overexpressed RAW264.7 cells (Fig. 7 A). We found that knockdown of Src and PKC ζ could block CHIP overexpression-induced activation of NF- κ B reporters after LPS/CpG treatments (Fig. 7 B), and the nuclear translocation of IRF3 and IRF7 were also decreased after knockdown of Src and PKC ζ (Fig. 7 C). These data suggest that Src and PKC ζ were both required for CHIP-mediated effects in TLR4/9 response.

Figure 4. CHIP binds HSC70, Src and PKC ζ .

(A) Wild-type RAW264.7 cells were treated with 100 ng/ml LPS, 10 μ g/ml poly (I:C), or 1 μ M CpG ODN, as indicated, and whole-cell lysates (WCL) were prepared. TLR4, TLR9, and TLR3 were immunoprecipitated (IP) with corresponding antibody plus protein A/G beads, and then components in immune complex were examined by Western blot. (B) GST fusion proteins were confirmed by immunoblot (IB) using GST antibody (top). GST pull-down assays were performed using lysates (WCL) from RAW264.7 cells (middle) or recombinant proteins (bottom). *, 30 μ g lysates; **, 1 ng recombinant proteins. (C) HEK293 cells were co-transfected with plasmids encoding HA-tagged CHIP or mutants and plasmids encoding Flag-tagged target molecules. 48 h later, immunoprecipitation assays were performed using anti-HA agarose or IgG agarose (*). The data shown correspond to a representative experiment out of three performed.



We have shown that CHIP could promote the kinase activity of IRAK1 and TBK1 (Fig. 6, B–E). However, the mechanisms responsible for Src/PKCζ activation-induced enhancement of IRAK1 and TBK1 activities were not revealed. Therefore, we examined the effects of Src/PKCζ knockdown on CHIP-mediated IRAK1 and TBK1 activation after LPS treatments. Considering that Src is a tyrosine kinase and that PKCζ is a serine/threonine kinase, we examined the phosphorylation of IRAK1 and TBK1 using phosphotyrosine-, phosphoserine-, and phosphothreonine-specific antibodies. We found that knockdown of Src/PKCζ could inhibit CHIP-mediated increase in the phosphorylated levels of IRAK1 and TBK1 (Fig. 7 D), indicating that CHIP may promote the activation of Src/PKCζ, which, in turn, phosphorylated and activated IRAK1/TBK1.

CHIP colocalizes with and polyubiquitinates Src and PKCζ

The N terminus of CHIP contains a myristoylation site. We found that CHIP was mainly localized within (EEA1-positive) endosomes, whereas the CHIPΔN20 (deletion of

Figure 5. CHIP knockdown inhibits TLR4/9-induced recruitment and activation of Src and PKCζ. (A) RAW264.7 cells stably transfected with CHIP-specific RNAi (RNAi) or scrambled control RNAi (CTRL) were treated with 100 ng/ml LPS or 1 μM CpG ODN, as indicated. TLR4 and TLR9 were immunoprecipitated (IP) with corresponding antibody plus protein A/G beads. Next, components in immune complex were examined by Western blot. WCL, whole-cell lysates. (B) Cell lysates derived from cells in A were immunoprecipitated with Src or PKCζ antibody or IgG plus protein A/G beads. Activation of Src (p-Src, phospho-Src) and PKCζ (p-PKCζ, phospho-PKCζ) was evaluated by immunoblot (IB) using phospho-specific antibodies. (C) RAW264.7 cells stably transfected with mock control vector or HA-tagged CHIP vector (CHIP-HA), pretreated with or without 10 nM Src inhibitor (PP1) or 10 μM PKCζ pseudosubstrate peptide (PS) for 30 min, were treated with 100 ng/ml LPS or 1 μM CpG ODN, as indicated. Activation of Src and PKCζ were evaluated as described in B. The data shown correspond to a representative experiment out of three performed.

N-terminal myristoylation signal) mutant was mainly localized in cytosol of RAW264.7 cells (Fig. 8 A). In LPS-treated RAW264.7 cells, CHIP was partially colocalized with TLR4, Src, and PKCζ (Fig. 8 B, rows 1–3), suggesting that CHIP may recruit Src and PKCζ onto endosomes during TLR4 response. Additionally, we found that Src and PKCζ could be recruited to TLR4 after LPS treatments (Fig. 8 B, rows 4 and 5).

Because CHIP is a U box-containing E3, we examined the ubiquitination status of Src and PKCζ in CHIP-silenced RAW264.7 cells and found that CHIP knockdown inhibited LPS-induced K63-linked polyubiquitination of both Src and PKCζ (Fig. 9, A and B). To verify the effects of E3 activity of CHIP on Src and PKCζ, we performed in vitro polyubiquitination assays in the presence of two popular E2s, UbcH5A, and UbCH13/Uev1A. We found that GST-CHIP could mediate the polyubiquitination of Src and PKCζ in the presence of both E2s (Fig. 9 C). To verify the ubiquitination forms of Src and PKCζ by CHIP, we applied recombinant ubiquitin containing K63 only (UbK63O) or ubiquitin containing K48 only (UbK48O) in the assays. We found that CHIP could mediate both K48-linked and K63-linked polyubiquitination of Src and PKCζ in vitro (Fig. 9 C).

Next, we determined the potential ubiquitinated sites in Src/PKCζ that were modified by CHIP. First, we predicted the ubiquitination sites by using the Bayesian discriminant method-prediction of ubiquitination sites algorithm. Second,

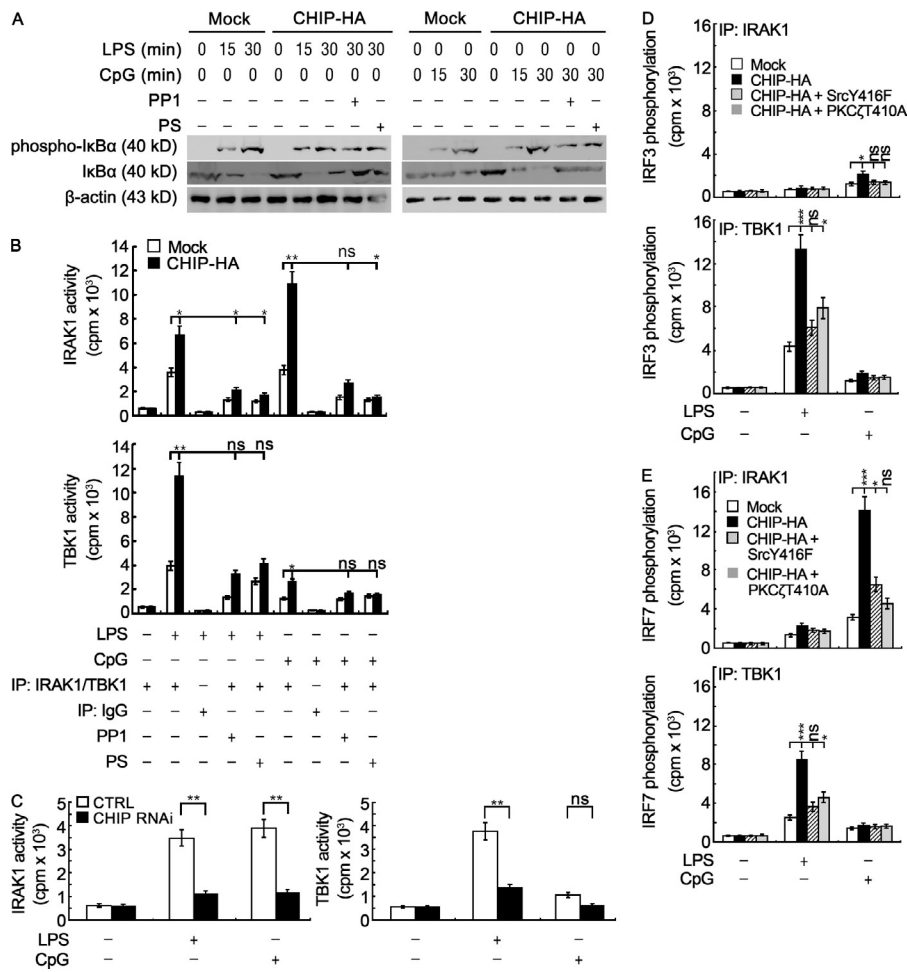


Figure 6. CHIP promotes TLR-triggered Src- and PKC ζ -dependent activation of I κ B α , IRAK1, and TBK1.

(A) Western blot assays of signaling mediators in RAW264.7 cells stably transfected with mock control vector or HA-tagged CHIP vector (CHIP-HA) and treated with 100 ng/ml LPS or 1 μ M CpG ODN, as indicated. Otherwise, cells were pre-treated with 10 nM Src inhibitor (PP1) or PKC ζ 10 μ M pseudosubstrate peptide (PS) for 30 min before LPS or CpG stimuli.

(B and C) In vitro kinase assays of IRAK1 and TBK1. In B, RAW264.7 cells stably transfected with mock control vector or HA-tagged CHIP vector (CHIP-HA) were used. In C, RAW264.7 cells stably transfected with scrambled control RNAi (CTRL) or CHIP-specific RNAi (RNAi) were used. These cells were treated with 100 ng/ml LPS or 1 μ M CpG ODN for 30 min. Kinase activity of immunoprecipitated (IP) IRAK1 and TBK1 was evaluated by in vitro kinase assays in the presence of 1 μ Ci of [γ - 32 P]-ATP (6,000 Ci/mmol) and 0.5 μ g of MBP as substrate. (D and E) In vitro kinase assays of IRAK1 and TBK1. Cells in A were transiently transfected with mock vector or Flag-tagged SrcY416F or PKC ζ T410A mutant vector for 48 h. After treatment with 100 ng/ml LPS or 1 μ M CpG ODN for 30 min, the kinase activity of immunoprecipitated (IP) IRAK1 and TBK1 was evaluated by in vitro kinase assays in the presence of 1 μ Ci of [γ - 32 P]-ATP (6,000 Ci/mmol) and 0.5 μ g of IRF3 (D) or IRF7 (E) as substrates. In B–E, results were presented as mean radioactivity \pm SD of triplicate samples. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data shown correspond to a representative experiment out of three performed.

we performed multiple alignment of Src/PKC ζ protein sequences derived from various species (for evolution conservation; unpublished data). By these 2 rounds of selection, Src may contain 20 ubiquitination sites, and PKC ζ may contain 9 ubiquitination sites (Fig. 9 D). To verify these ubiquitination sites, we constructed 12 mutants for Src and 6 mutants for PKC ζ , and examined the ubiquitin levels associated with Src/PKC ζ after LPS treatments in RAW264.7 cells using ELISA. We found that Src-K5/7/9R and Src-K324/329R mutations could most significantly abolish the polyubiquitinated Src levels, whereas PKC ζ -K220/225R could effectively block the polyubiquitinated levels of PKC ζ (Fig. 9 E). These data suggest that K5/7/9 and K324/329 in Src, and K220/225 in PKC ζ , may be the major ubiquitination sites modified during TLR4 response.

To verify whether these sites were modified by CHIP, we transfected these mutants into CHIP-overexpressed cells. We found that polyubiquitination of Src-K5/7/9R, Src-K324/329R, and PKC ζ -K220/225R was impaired, as compared with wild-type Src or PKC ζ (Fig. 9 F). More importantly, Src-K5/7/9R, Src-K324/329R, and PKC ζ -K220/225R transfection could block the effects of CHIP in activating NF- κ B and IFN- β reporters (Fig. 9, G and H). Therefore, polyubiquitination of Src

and PKC ζ by CHIP may be crucial in CHIP-mediated effects on regulating TLR response.

To look into the effects of localization and E3 activity of CHIP, we stably overexpressed CHIP Δ N20 and CHIPH261Q (E3 activity deficiency) in RAW264.7 cells. We found that LPS and CpG-induced kinase activities of Src and PKC ζ were significantly decreased (Fig. S7 A). Interestingly, we found that production of IL-6 and IFN- β was significantly inhibited in RAW264.7 cells stably overexpressing CHIP Δ N20 and CHIPH261Q (Fig. S7 B). These data together suggest that CHIP-mediated effects in TLR response may require both the endosomal localization and the E3 activity of CHIP.

CHIP promotes DC maturation

In BMDCs, knockdown of CHIP decreased the recruitment of Src and PKC ζ to the TLR4/9 complex (Fig. S8 A), impaired the kinase activity of Src and PKC ζ (Fig. S8 B), inhibited the kinase activity of IRAK1 and TBK1 (Fig. S8 C), and attenuated the expression of IL-12p40 and CD40 (Fig. 10 A). These data confirm that CHIP-mediated recruitment and

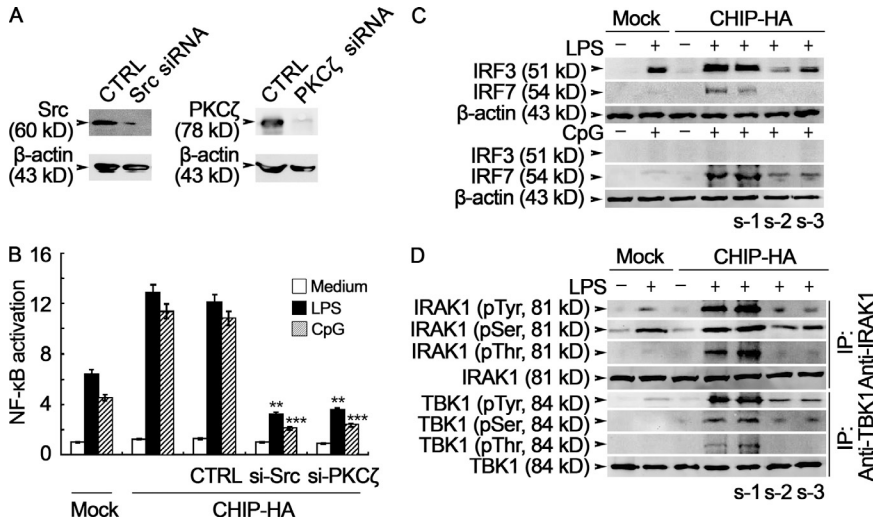


Figure 7. Knockdown of Src and PKC ζ blocks the effects of CHIP in TLR response.

(A) RAW264.7 cells stably transfected with mock vector or CHIP-HA vector were transiently transfected with control siRNAs (CTRL, for GFP) or Src/PKC ζ -specific siRNAs for 48 h. The efficiency of silencing was evaluated by Western blot. (B) Cells in A were transiently transfected with NF- κ B reporters for 48 h. Cells were treated with 100 ng/ml LPS or 1 μ M CpG ODN for 4 h. Results were expressed as fold induction relative to the activity in unstimulated cells transfected with mock vector. Data are shown as mean \pm SD of triplicate samples. si-Src, siRNAs specific for Src; si-PKC ζ , siRNAs specific for PKC ζ ; ns, not significant; **, $P < 0.01$; ***, $P < 0.001$ (as compared with corresponding cells without LPS/CpG treatments). (C and D) Cells in A were treated with 100 ng/ml LPS for 1 h (C) or 30 min (D), and then nuclear extracts (C) and cell lysates (D) were prepared. Nuclear translocation of IRF3/7 (C) or phosphorylated IRAK1/TBK1 within immunoprecipitates (IP; D) was examined by Western blot. s-1, control siRNAs for GFP; s-2, Src-specific siRNAs; s-3, PKC ζ -specific siRNAs. The data shown correspond to a representative experiment out of three performed.

activation of Src and PKC ζ are also required for DC activation and maturation upon TLR4/9 signaling.

Type I IFN-mediated signaling play important roles in maturation of DCs (Gautier et al., 2005; Mattei et al., 2009). Thus, to examine the effects of CHIP in LPS/CpG-induced maturation of DCs, we measured the production of IFN- β in BMDCs after CHIP overexpression. We found that CHIP could significantly increase the levels of IFN- β , whereas CHIP Δ N20 and CHIPH261Q could inhibit the production of IFN- β after LPS/CpG treatments (Fig. 10 B). Moreover, Src inhibitor PP1, PKC ζ pseudosubstrate, kinase inactive form of Src, and PKC ζ could block CHIP-mediated increase in IFN- β production (Fig. 10 B).

To further elucidate the roles of CHIP in LPS/CpG-induced maturation of DCs, we used the BMDC-derived from IFNAR $^{-/-}$ mice. We found that CHIP overexpression in IFNAR $^{-/-}$ BMDCs failed to promote LPS/CpG-induced maturation of DCs (Fig. 10, C and D), which indicated that CHIP-mediated type I IFN production was required for LPS/CpG-induced DC maturation.

DISCUSSION

Here, we have identified the function and the underlying mechanisms of CHIP in the regulation of TLR signaling in APC. We demonstrate that CHIP can orchestrate the TLR4/9 signaling pathway by recruiting and activating Src/PKC ζ , enhancing kinase activity of IRAK1/TBK1 and promoting degradation of I κ B α , which lead to activation of NF- κ B and IRF3/7 and activation/maturation of macrophages and DCs. Thus, CHIP may be the first U box-containing E3 identified in mammalian TLR response. Previous studies have identified CMPG1 as a potential regulator of plant defense machinery (González-Lamothe et al., 2006) and Act1/CIKS has been shown to interact with TRAF6 (Kanamori et al., 2002). However, these U box-containing E3s have not been investigated for their roles in TLR response in APCs. Our study suggests that CHIP, the endosome-associated U box-containing E3, may be a scaffold molecule orchestrating the assembly of TLR4/9 and

possibly TLR2/7 complexes, but not TLR3 complex, within endosomes. Our data suggest that CHIP knockdown doesn't affect the TLR3 recruitment of Src and other signaling molecules and the activation of IRF3-IFN- β by poly(I:C), indicating that CHIP may be dispensable for TLR3 signaling. One possibility is that TLR3 may directly recruit Src, whereas TLR4/9 may recruit Src via a scaffold protein. The differences between CHIP-mediated effects on regulation of TLR4/9 signaling versus TLR3 signaling may thus need further investigations. Moreover, the CHIP-mediated effects on TLR response may need to be further verified in CHIP $^{-/-}$ mice, which are now commercially unavailable, and the possibility that CHIP-mediated regulation of TLR response may be involved in CHIP knockout-induced atrophy of multiple organs and cell apoptosis needs additional study. However, our data demonstrate that stable silence of CHIP in RAW264.7 cells and transient knockdown of CHIP in BMDCs and pDCs can inhibit TLR2/4/7/9 signaling-triggered cytokine production and TLR4/9-induced activation of Src-PKC ζ -IRAK1-TBK1-IRF3-NF- κ B signaling pathway, which strongly and clearly outline the signaling mechanisms of CHIP in TLR response.

Endosomes are the major sites of TLR complex assembly (Kawai and Akira, 2010; McGettrick and O'Neill, 2010). It has been shown that TLR4 in endosomes may associate with TRIF-TBK1 and regulate IFN- α/β production (Kagan et al., 2008). More typically, TLR7/9 and TLR3 have been suggested to initiate antiviral response and type I IFN production in endosomes or endolysosomes (Kawai and Akira, 2010; McGettrick and O'Neill, 2010). Therefore, CHIP-mediated assembly of TLR complex may be an essential mechanism for TLR-triggered type I IFN production. However, our study suggests that CHIP may be not involved in

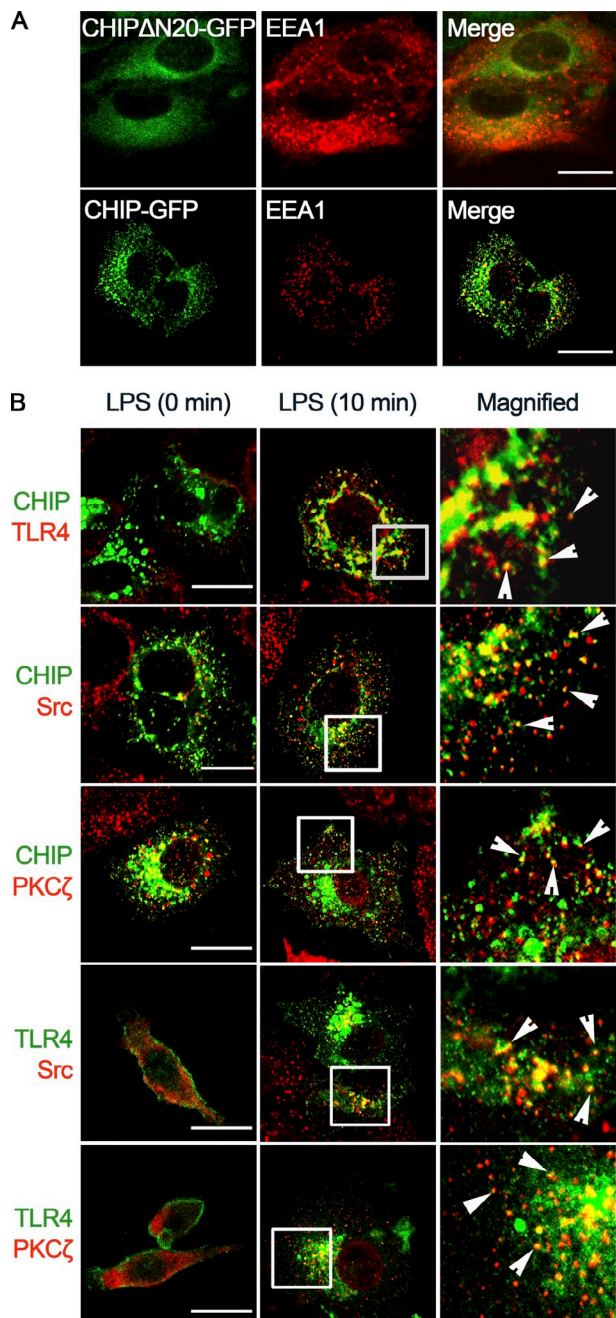


Figure 8. CHIP colocalizes with Src and PKC ζ . (A) Localization of CHIP in endosomes. RAW264.7 cells were transiently transfected with GFP-tagged CHIP Δ N20 or CHIP vector for 48 h. Cells were stained with anti-EEA1 and Alexa Fluor 555-conjugated secondary antibody. Bars, 100 μ m. (B) RAW264.7 cells were transiently transfected with CHIP-GFP for 48 h, and treated with 100 ng/ml LPS for 10 min (rows 1–3). Cells were stained with anti-TLR4, Src, PKC ζ antibodies, and Alexa Fluor 555-conjugated secondary antibody. RAW264.7 cells were treated with 100 ng/ml LPS for 10 min (rows 4 and 5). Cells were stained with anti-TLR4, Src, or PKC ζ antibodies, and Oregon Green 488-conjugated or Alexa Fluor 555-conjugated secondary antibody. In B, the merged images are shown. The boxed regions in the middle image were correspondingly magnified and shown on the right. White arrow heads indicated for the typical colocalization sites. Bars, 100 μ m. The data shown correspond to one representative experiment out of three performed.

TLR3 complex formation, indicating that TLR2/4/7/9 complex components may be different to those of TLR3. The observation that HSC70 is not immunoprecipitated with TLR3 may suggest that CHIP recruitment to TLR complex may be through HSC70, which may be the reason for CHIP inability to regulate TLR3 response. One potential inconsistency in our study is the observation that CHIP is a component of both TLR4–MyD88–IRAK1 and TLR4–TRIF–TBK1 complexes (Kawai and Akira, 2010; McGettrick and O’Neill, 2010). The assembly of TLR4 complex mainly occurs at the proximal intracellular region of TLR4 that recruits MyD88 and MyD88-associated molecules, which may activate NF- κ B and MAPK to initiate proinflammatory cytokine production; although after internalization, TLR4 can complex with TRIF and TRIF-associated molecules, which may activate TBK1–IRF3/7 to initiate type I IFN production (Kagan et al., 2008; Kawai and Akira, 2010; McGettrick and O’Neill, 2010). In our study, we found that CHIP can potentiate the effects of LPS and CpG in the production of IFN- β and IL-6, indicating that a portion of TLR4 complex formation may also exist in endosomes. Considering that plasma membrane TLR4 can be translocated from cell surface to endosomes rapidly (within 10 min; Wang et al., 2007), CHIP-mediated effects in TLR response may take place in endosomes. However, it remains to be determined whether TLR4–MyD88–IRAK1 assembly partially occurs within endosomes.

LPS-initiated, MyD88-dependent signaling pathways can activate MAPK and NF- κ B via sequential activation of MyD88–IRAK–TRAF6, resulting in production of proinflammatory cytokines (IL-1, IL-6, TNF α etc.; Medzhitov et al., 1998; Takaesu et al., 2000). LPS can also initiate TRIF-dependent activation of IRF3/7 to regulate the IFN-inducible genes (CXCL10, IFN- α/β , etc.; Fitzgerald et al., 2003; Sharma et al., 2003; Jiang et al., 2004; Kawai and Akira, 2010; McGettrick and O’Neill, 2010). Our study demonstrates that CHIP can regulate the IL-6 production and IFN- β production induced by TLR4/9. We also show that CHIP modulates the activation of IRAK1 and TBK1 through Src and PKC ζ . Therefore, the effects of CHIP in TLR response may rely on activation of Src and PKC ζ , which are confirmed by the experiments that Src/PKC ζ inhibitors, dominant-negative Src/PKC ζ , and Src/PKC ζ siRNAs impaired the effects of CHIP in regulating TLR response.

Protein tyrosine kinases, such as Btk, Syk, and Src, have been reported to regulate TLR response (Kawai and Akira, 2010; McGettrick and O’Neill, 2010). It has been shown that Btk can phosphorylate TLR4 and Mal, which are required for TLR4-triggered NF- κ B activation (Jefferies et al., 2003; Gray et al., 2006). Src inhibitors can inhibit TLR4 and TLR9 signaling (Stovall et al., 2004; Medvedev et al., 2007). Syk has been shown to regulate TLR signaling pathways (Zhang et al., 2009). For the Src family of tyrosine kinases, it has been demonstrated that Src can potentiate both TLR4 and TLR3 signaling (Smolinska et al., 2008; Kuka et al., 2010). Src has also been implicated in the TRIF–TBK1–IRF3 complex during poly(I:C)-induced TLR3 signaling (Johnsen et al., 2006).

For atypical PKC ζ/ι , evidence suggests that they are involved in the regulation of TLR response (Monick et al., 2000; Cuschieri et al., 2004; Teusch et al., 2004). It has been shown that PKC ι can directly phosphorylate IRAK1 in Thr66 and promote autophosphorylation of IRAK1 (Mamidipudi et al., 2004). Therefore, current data suggest a potential involvement of Src and PKC ζ in TLR response by regulating phosphorylation and activity of IRAK1 or TBK1. More importantly, an

interplay exists between Src and atypical PKCs, and Src can mediate the tyrosine phosphorylation of PKC ζ (Wooten et al., 2001). Our study suggests that CHIP-mediated Src and PKC ζ activation may lead to activation of IRAK1 and TBK1, which has been confirmed by using Src/PKC ζ inhibitors, dominant-negative Src/PKC ζ , and Src/PKC ζ siRNAs. Given that IRAK1 can activate IRF7 and that TBK1 can activate IRF3/7 (Sharma et al., 2003; Uematsu et al., 2005), it may be

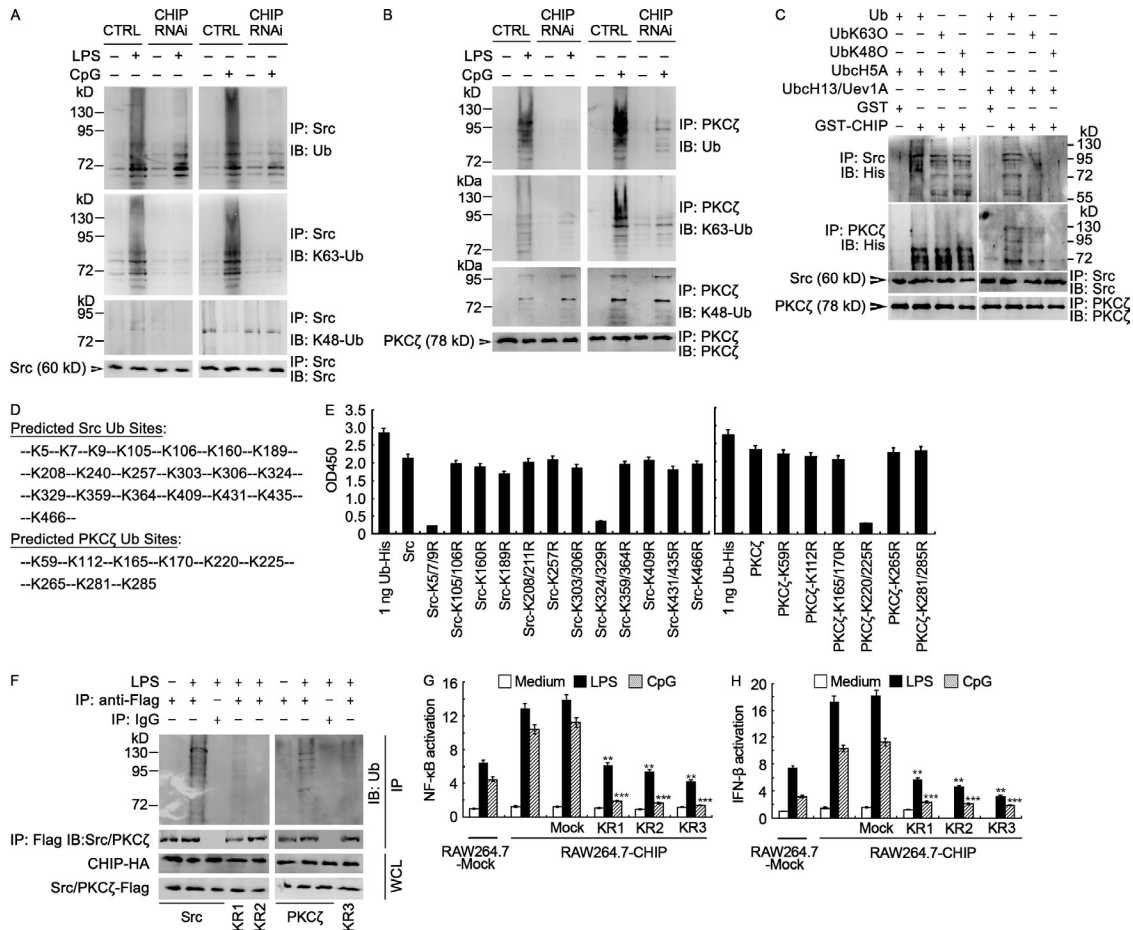


Figure 9. CHIP polyubiquitinates Src and PKC ζ . (A and B) In vivo polyubiquitination assays. RAW264.7 cells stably transfected with scrambled control RNAi (CTRL) or CHIP-specific RNAi (RNAi) were treated with 100 ng/ml LPS or 1 μ M CpG ODN for 30 min. Src and PKC ζ contained in lysates were then subjected to heat denaturing in 1% SDS and immunoprecipitated (IP) with Src (A) or PKC ζ (B) antibody plus protein A/G agarose. Ubiquitination was examined by immunoblot (IB) using anti-ubiquitin (Ub), anti-K63-Ub, or anti-K48-Ub antibody as indicated. (C) In vitro polyubiquitination assays. GST-CHIP was incubated with recombinant Src or PKC ζ in the presence of E1, His6-Ub derivatives, and UbcH5A or Ubc13/Uev1A E2/Ubc as indicated. Src and PKC ζ were immunoprecipitated (IP) with anti-Src or anti-PKC ζ antibody after heat denaturing in 1% SDS. UbK63O, ubiquitin containing only Lys63; UbK48O, ubiquitin containing only Lys48. (D) Predicted ubiquitination sites in Src and PKC ζ using the Bayesian discriminant method-prediction of ubiquitination sites algorithm, as well as protein sequence alignment. (E) RAW264.7 cells were transiently transfected with Flag-tagged wild-type or mutant Src (left) or PKC ζ (right) constructs for 48 h, and then treated with 100 ng/ml LPS for 30 min. Ubiquitin levels associated with Src/PKC ζ (mutants) were evaluated by ELISA at 450 nm. 1 ng recombinant ubiquitin-His was used as positive control. (F) RAW264.7 cells stably transfected with CHIP-HA were transiently transfected with Flag-tagged wild-type or mutant (KR1-3) Src or PKC ζ constructs for 48 h, and then treated with 100 ng/ml LPS for 30 min. Src and PKC ζ contained in lysates were immunoprecipitated (IP) with Flag agarose or IgG plus protein A/G agarose as indicated. Ubiquitination was examined by immunoblot (IB) using anti-Ub antibody. (G and H) RAW264.7 cells stably transfected with mock vector (RAW264.7-Mock) or CHIP-HA vector (RAW264.7-CHIP) were transiently transfected with Flag-tagged Src and PKC ζ mutants (KR1-3) and NF- κ B (G) or IFN- β (H) reporters for 48 h, and then treated with 100 ng/ml LPS or 1 μ M CpG ODN for 4 h. Data are shown as mean \pm SD of triplicate samples. **, P < 0.01; ***, P < 0.001 (as compared with corresponding RAW264.7-CHIP cells transiently transfected with mock vector and treated with LPS/CpG). In F-H, KR1 indicates for Src-K5/7/9R, KR2 for Src-K324/329R, and KR3 for PKC ζ -K220/225R. The data shown in A-C and E-H correspond to a representative experiment out of three performed.

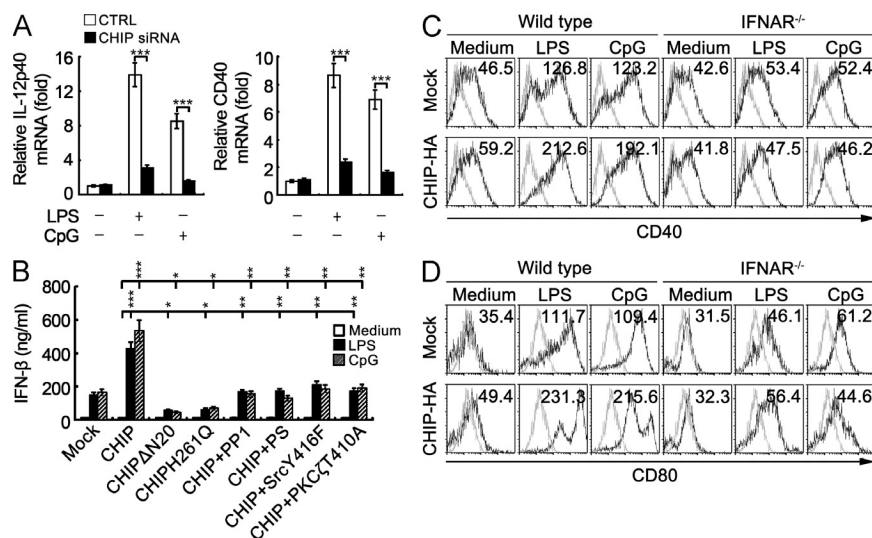


Figure 10. CHIP enhances TLR4- and TLR9-triggered DC maturation via IFN- α/β -dependent pathway. (A) 48 h after transient transfection of siRNAs in day 6 BMDCs, the cells were treated with 100 ng/ml LPS or 1 μ M CpG ODN for 4 h. The mRNA levels of IL-12p40 and CD40 were evaluated by quantitative PCR. (B) Day 6 BMDCs were transiently co-transfected with mock vector, CHIP-HA or CHIP mutant-HA vectors, and SrcY416F or PKC ζ T410A vector as indicated for 48 h, and then treated with 100 ng/ml LPS or 1 μ M CpG ODN for 8 h in the presence or absence of 10 nM Src inhibitor (PP1) or 10 μ M PKC ζ pseudosubstrate peptide (PS). IFN- β levels were measured by ELISA. In A and B, results were presented as mean \pm SD of triplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C and D) Day 6 BMDCs derived from wild-type or IFNAR^{-/-} mice were transiently transfected with mock vector or CHIP-HA vector for 48 h, and treated with 100 ng/ml LPS or 1 μ M CpG ODN for 48 h. The expression levels of DC markers CD40 (C) and CD80 (D) were evaluated by FACS assays, and the numbers in each panel indicate mean fluorescence intensity. The data shown correspond to one representative experiment out of three performed.

inferred that CHIP-mediated production of IFN- β may be through Src/PKC ζ -dependent activation of IRAK1/TBK1. However, one question still remains: how are IRAK1 and TBK1 activated by Src/PKC ζ ? Detailed examination of phosphorylated sites in IRAK1 and TBK1 affected by Src/PKC ζ may help to reveal the mechanisms.

However, we have not satisfactorily explained the effects of CHIP on NF- κ B activation. We show that CHIP cannot affect TLR4/9-induced phosphorylation of IKK α/β on Ser176/180 and I κ B α on Ser32/36, but promote the degradation of I κ B α . Previously, Src and PKC ζ have been implicated in the regulation of NF- κ B by tyrosine or Ser/Thr phosphorylation of IKK α/β , respectively (Leitges et al., 2001; Huang et al., 2003; Chang et al., 2004). The lack of increased phosphorylation of I κ B α on Ser32/36 may suggest that CHIP-mediated activation of Src/PKC ζ may not directly promote the phosphorylation of IKK α/β . Two possibilities may exist for CHIP-mediated activation of NF- κ B. Src and PKC ζ may directly activate NF- κ B through I κ B α phosphorylation, which is supported by the report that Src can mediate tyrosine phosphorylation and degradation of I κ B α (Koong et al., 1994). Another possibility is that Src and PKC ζ directly activate p50/p65 subunits, which is supported by the findings that Src and PKC ζ can directly phosphorylate p65 (Savkovic et al., 2003; Bijli et al., 2007). However, the final detailed mechanisms of CHIP-mediated activation of NF- κ B (especially in TLR4 signaling) need further investigations.

We have shown that CHIP is an E3 for Src and PKC ζ . Our data suggest that CHIP can ubiquitinate both Src and PKC ζ mainly through K63-linked manner. Considering that K63-linked polyubiquitination can regulate the activity of substrate and the assembly of molecular complexes, we suggest here that CHIP may regulate the activity of Src and PKC ζ by K63-linked polyubiquitination. Supporting this proposal, it has been reported that the crystal structure of CHIP indicates for a CHIP-Ubc13-Uev1A complex (Zhang et al., 2005). Therefore, CHIP may regulate the proximal

assembly of TLR response by linking K63-linked polyubiquitinated Src and PKC ζ to TLR signaling components, e.g.,

TRAF6 and p62/SQSTM1 (Samuels et al., 2001). However, the detailed effects of CHIP on the formation of TLR complex may need further investigations.

MATERIALS AND METHODS

Mice and reagents. IFNAR^{-/-} mice were obtained from The Jackson Laboratory. All the animal experiments were approved by the Medical Ethics Committee of the Zhejiang University School of Medicine and conducted according to the Declaration of Helsinki Principles. Pam3Cys, R848, Poly(I:C), and LPS (0111:B4) were purchased from Sigma-Aldrich. Phosphorothioate-modified CpG ODN synthesized by Sybersyn and LPS were repurified as previously described (Wang et al., 2007). Antibodies specific to HA-tag, His-tag, Flag-tag, HSC70, TRAF6, IRF3 and ubiquitin, the recombinant IRF3/7, Src and PKC ζ proteins, and the agaroses used in immunoprecipitations were obtained from Abcam. Antibodies specific for TLR4, TLR9, IRAK1, TBK1, TRIF, MyD88, IRF7, phospho-Tyr, phospho-Ser and phospho-Thr, and antibodies specific for total and phosphorylated forms of ERK1/2 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), p38 (Thr180/Tyr182), IKK α/β (Ser176/180), Src (Tyr416), PKC ζ (Thr410), and I κ B α (Ser32/36) were purchased from Cell Signaling Technology. Antibodies for β -actin and CHIP were obtained from Sigma-Aldrich. Recombinant MBP, and antibodies against K48-ubiquitin and K63-ubiquitin, were purchased from Millipore. Ubiquitin and derivatives were purchased from Boston Biochem. The pGL3.5X-B-luciferase plasmid was a gift from S.J. Martin (Smurfit Institute, Dublin, Ireland; Bouchier-Hayes et al., 2001) and the pRL-TK-Renilla-luciferase plasmid was obtained from Promega. IRF3 reporter plasmids were a gift from T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; Shinobu et al., 2002). Fluorescent antibodies used in confocal microscopy were purchased from Invitrogen. siRNAs used in this study for indicated E3s were obtained from Santa Cruz Biotechnology, Inc.

Construction of expression plasmids. The recombinant vectors encoding mouse CHIP (available from GenBank/EMBL/DDBJ under accession no. NM_019719), HSC70 (accession no. NM_031165), Src (accession no.

NM_009271), PKC ζ (accession no. NM_008860), and TRAF6 (accession no. NM_009424) were constructed by PCR-based amplification, and then subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen). Vectors encoding HA-tagged CHIP (termed as CHIP-HA), GFP-tagged CHIP (termed as CHIP-GFP), or Flag-tagged HSC70, Src, PKC ζ , and TRAF6 were constructed and prepared as previously described (Wang et al., 2007). Mutant forms of CHIP, namely CHIPK31A (mutation of Lys31 into Asn, HSC70-binding deficiency), CHIPH261Q (mutation of His261 into Gln, E3 activity deficiency), CHIP Δ TPR (truncation of the N-terminal tetrapeptide repeat), CHIP Δ U (truncation of the C-terminal U box domain), and CHIP Δ N20 (deletion of the N-terminal myristylation sequence) were prepared by using MutanBest kit according to the manufacturer's instructions (TaKaRa Bio Inc.). All the clones were confirmed by DNA sequencing. Primer sequences used in cloning are available upon request.

Cell preparation, cell culture, and transfection. RAW264.7 macrophages, HEK293 cells, HEK293 cells stably transfected with mock vector or TLR3 vector (Invitrogen), and primary peritoneal macrophages were obtained or prepared as previously described (Wang et al., 2007; Wang et al., 2009). MEFs deficient for MDA-5 were gifts from S. Akira (Research Institute for Microbial Diseases, Osaka, Japan). BMDCs were generated by culturing C57BL/6 BM cells for 10 d (pDCs) in medium containing 40 ng/ml Flt-3 ligand (R&D Systems) or for 6 d (BMDCs) in medium containing 20 ng/ml recombinant GM-CSF and 10 ng/ml IL-4 (Genzyme), as previously described (Chen et al., 2004; He et al., 2011). CD11c⁺ BMDCs were isolated using MACS cell separation reagents (Miltenyi Biotec), and CD11c⁺B220⁺ pDCs were sorted by using FACS Vantage flow cytometer (BD). For transient transfection of plasmids in RAW264.7 cells, jetPEI reagents were used (Polyplus Transfection Company). For transient transfection of BMDCs, jetPEI-Macrophage reagents were used (Polyplus Transfection Company). Stable cell lines overexpressing CHIP and its mutants were selected in 600 μ g/ml G418 for 3–4 wk.

RT-PCR and quantitative PCR. Total cellular RNA was extracted using TRIzol reagent (Invitrogen). RT-PCR was performed as previously described (Chen et al., 2004). Specific primers used for RT-PCR assays were 5'-GTGCGCAAGAGCTCAAGGAGC-3' (sense) and 5'-GCCGTTCTCAGAGATGAAAGCG-3' (antisense) for CHIP, and 5'-AGTGTGACGTTGACATCCGT-3' (sense) and 5'-GCAGCTCAGTAACAGTCCGC-3' (antisense) for β -actin. Quantitative PCR was performed on a MJR Chromo4 Continuous Fluorescence Detector (Bio-Rad Laboratories) according to the manufacturer's protocol and as previously described (Wang et al., 2007).

RNA interference. We have synthesized and selected four siRNA duplexes (siRNA1–4; Shanghai GenePharma Co.). For transient transfection, 21-nt sequences of CHIP siRNA (siRNA4) were synthesized as follows: 5'-AUACAUGGCAGAUUGGAUUU-3' (sense), and 5'-AAAUCCAUAUCUGCCAUGUAU-3' (antisense). The sequences 5'-AUACAUGCCAGAUUGCAUUU-3' (sense) and 5'-AAAUGCAUAUCUGGCAUGUAU-3' (antisense) were used as a scrambled RNA interference control (CTRL). siRNA duplexes were transfected into macrophages and DCs using INTERFERin-HTS according to the standard protocol (Polyplus Transfection Company). For stable knockdown of CHIP, an expression vector (psilencer-U6 neo; Invitrogen) with insertion of the specific siRNA duplexes of CHIP or the scrambled siRNA duplexes were transfected into RAW264.7 cells (Wang et al., 2007). To exclude the possibility of off-target effects of the siRNA4, a plasmid (CHIP-R4) resistant to siRNA-4 was constructed and transfected into CHIP-silenced cells.

Measurement of cytokines. ELISA kits for mouse IL-6, TNF, and IFN- β were obtained from R&D Systems. Cytokine concentrations in culture supernatants were measured by ELISA as previously described (Wang et al., 2007).

Luciferase reporter assays. The IFN- β luciferase reporter plasmids were constructed by us as previously described (Wang et al., 2007). For construction of IL-6 and CCL5 reporter, DNA sequences (-256/+1 of IL-6 and -979/+8

of CCL5) were amplified from RAW264.7 genomic DNA by nested PCR, and the products were inserted into KpnI–Hind III sites of pGL3-Basic vector (Promega). All constructs were confirmed by DNA sequencing. The determination of reporter transactivation was performed as previously described (Wang et al., 2007; Wang et al., 2009).

Flow cytometry. For analysis of phenotypes of BMDCs after treatments, cells were stained with fluorescent antibodies (BioLegend) and analyzed by flow cytometry as previously described (Chen et al., 2004).

Western blotting and extraction of nuclear proteins. Total cell lysates were prepared as previously described (Wang et al., 2007), and protein concentration was determined by the BCA protein assay (Thermo Fisher Scientific). Nuclear proteins were extracted by NE-PER Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell extracts were subjected to SDS-PAGE, transferred onto nitrocellulose membrane, and blotted as previously described (Chen et al., 2004).

Electromobility shift assay (EMSA) of NF- κ B. Nuclear proteins were extracted using NE-PER nuclear extraction reagents (Thermo Fisher Scientific). The EMSAs were performed using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the manufacturer's instructions and as previously described (He et al., 2011).

Immunoprecipitation. The immunoprecipitation and the immunoblot assays were performed as previously described (Wang et al., 2009).

GST pull-down assays. The cDNAs encoding CHIP and indicated fragments of CHIP were cloned into pGEX-2T vector (GE Healthcare). The expression and purification of GST fusion proteins and the GST pull-down assays were performed as previously described (Wang et al., 2009).

In vitro kinase assays. For in vitro kinase assays, 100 μ g proteins contained in total cell extracts were immunoprecipitated with indicated antibodies plus protein A/G beads by gently rocking at 4°C for 2 h, followed by centrifugation at 4°C for 5 min. Next, the in vitro kinase activity assay kits for Src and PKC ζ from Cell Signaling Technology were used as instructed by the manufacturer. The kinase activity of IRAK1 and TBK1 was determined by measuring radioactive autophosphorylation of MBP or IRF3/7 as previously described (Wang et al., 2009).

Polyubiquitination assays. The in vitro and in vivo polyubiquitination assays were performed as previously described (Wang et al., 2009). To determine the polyubiquitination sites within Src and PKC ζ , the PDM-PUB algorithm was used (<http://bdmpub.biocuckoo.org/prediction.php>), and alignment of multiple Src/PKC ζ sequences of various species was performed to assess the evolution conservation of the predicted lysine residues. An ELISA method was used to verify these ubiquitination sites. Flag-tagged plasmids encoding indicated Src/PKC ζ mutants (lysine to arginine mutation) were transiently transfected into RAW264.7 cells, and then the cell lysates were prepared after LPS treatments using a buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 1% SDS. After heat denaturing for 5 min, Src/PKC ζ was immunoprecipitated with Flag agarose (Sigma-Aldrich) and eluted with RIPA buffer containing 0.2% SDS and 0.1% Tween 20. Next, the supernatants were subjected to ELISA assays. In brief, ultra-high-binding 96-well microtiter ELISA plates (Thermo Fisher Scientific) were coated overnight with rabbit polyclonal ubiquitin antibody (Abcam) in carbonate buffer, followed by three washes in PBS plus 0.1% Tween 20 (PBST; Sigma-Aldrich). The plates were blocked with 1% BSA in PBST, followed by three washes in PBST. Samples or recombinant ubiquitin proteins were added to the wells and incubated for 1 h, followed by three washes in PBST. Mouse monoclonal anti-ubiquitin (Sigma-Aldrich) was added to each well and incubated for 1 h, followed by three washes with PBST. Anti-mouse IgG HRP-conjugated antibody was

added to the 96-well plates, followed by three washes with PBST. Finally, the substrate TMB was added, and absorbance was read by spectrophotometer (Bio-Rad Laboratories) at 450 nm.

Immunofluorescence staining and confocal microscopy. RAW264.7 cells transiently transfected with plasmids encoding CHIP-GFP and CHIP Δ N20-GFP were cultured on coverslips for 48 h. For the colocalization analysis of CHIP or TLR4 with early endosome marker EEA1, TLR4, PKC ζ or Src, wild type or transfected RAW264.7 cells were immunostained with first antibody against EEA1, TLR4, PKC ζ or Src as indicated, and then with proper Oregon Green 488- or Alexa Fluor 555-conjugated secondary antibodies. The immunostaining process was performed as described (Wang et al., 2007). Slides were finally examined under a fluorescence confocal microscopy (LSM confocal microscope; Carl Zeiss, Inc.) as previously described (Wang et al., 2007).

Statistical analysis. All the experiments were independently repeated at least three times. Results are given as mean \pm SEM or mean \pm SD. Comparisons between two groups were done using Student's *t* test analysis. Multiple comparisons were done with a one-way ANOVA, followed by Fisher's least significant difference analysis, or done with Kruskal-Wallis tests. Statistical significance was determined as $P < 0.05$.

Online supplemental material. Fig. S1 shows the effects of silencing several E3 ligases or A20 DUB on production of TNF or IFN- β by DCs during TLR4 response. Fig. S2 shows the effects of transient transfections of four CHIP siRNAs, or the siRNA4-resistant CHIP mutant on CHIP expression and on TNF or IFN- β production by DCs during TLR4 response. Fig. S3 shows the expression pattern of CHIP in immune cells, the efficiency of CHIP siRNA on reducing CHIP expression, and the production of TNF by CHIP-silenced peritoneal macrophages after TLR ligands activation. Fig. S4 shows the effects of CHIP knockdown on IL-12p70 production and maturation of BMDCs. Fig. S5 shows the effects of CHIP silence on TLR3 signaling. Fig. S6 shows the effects or CHIP silence or CHIP overexpression on the kinase activity of Src and PKC ζ . Fig. S7 shows that the localization and E3 ligase activity of CHIP are required for its roles in TLR response. Fig. S8 shows that knockdown of CHIP impairs TLR4 and TLR9 response in BMDCs. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20102667/DC1>.

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