MARCH3 attenuates IL-1β–triggered inflammation by mediating K48-linked polyubiquitination and degradation of IL-1RI

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The proinflammatory cytokine IL-1β plays critical roles in inflammatory and autoimmune diseases. IL-1β signaling is tightly regulated to avoid excessive inflammatory response. In this study, we identified the E3 ubiquitin ligase membrane-associated RING-CH-type finger 3 (MARCH3) as a critical negative regulator of IL-1β–triggered signaling. Overexpression of MARCH3 inhibited IL-1β–triggered activation of NF-κB as well as expression of inflammatory genes, whereas MARCH3 deficiency had the opposite effects. MARCH3-deficient mice produced higher levels of serum inflammatory cytokines and were more sensitive to inflammatory death upon IL-1β injection or *Listeria monocytogenes* infection. Mechanistically, MARCH3 was associated with IL-1 receptor I (IL-1RI) and mediated its K48-linked polyubiquitination at K409 and lysosomal-dependent degradation. Furthermore, IL-1β stimulation triggered dephosphorylation of MARCH3 by CDC25A and activation of its E3 ligase activity. Our findings suggest that MARCH3-mediated IL-1RI degradation is an important mechanism for attenuating IL-1β–triggered inflammatory response.

MARCH3 | IL-1 | *Listeria monocytogenes* | polyubiquitination | inflammation

The proinflammatory cytokine IL-1β is a central regulator in the initiation of inflammatory and immune responses. It also plays critical roles in the pathogenesis of different diseases, such as cancer, rheumatoid arthritis, neurodegenerative diseases, and atherosclerosis (1–3). The initial step in IL-1β signal transduction is a ligand-induced conformational change in the first extracellular domain of the IL-1 receptor I (IL-1RI) that facilitates recruitment of IL-1 receptor accessory protein (IL-1RAcP) (4). The activated receptor complex can then recruit the adaptor protein MyD88 via Toll-like/IL-1R (TIR)–TIR domain interactions (5). MyD88 further recruits the two kinases IRAK1 and IRAK4, and TRAF6 to the receptor complex (5–9), where TRAF6 catalyzes K63-linked autoubiquitination to further recruit the TAK1–TAB2–TAB3 complex, resulting in the activation of TAK1. Subsequently, TAK1 activates IKKs and MAPKs, leading to activation of the transcription factors NF-κB and AP1, induction of downstream effector genes, and inflammatory responses (10–12).

While IL-1β–triggered signaling plays important roles in host defense to pathogens, deregulation or excessive activation of the signaling events causes dangerous and detrimental local or systemic inflammatory reactions, as well as autoimmune or allergic responses (3, 13). Thus, the IL-1β–triggered signaling is tightly regulated by diverse mechanisms to avoid host damage. It has been reported that IL-1Ra acts as an IL-1RI antagonist and competes with IL-1β for receptor binding (14), whereas IL-1RII lacks a signaling domain and acts as a decoy and dominant negative receptor for IL-1β (15). Several proteins have been reported to modulate crucial components involved in IL-1β–triggered signaling pathways. MARCH8 catalyzes K48-linked polyubiquitination and degradation of IL-1RAcP (16). Pellino 3b acts as a negative regulator for IL-1β signaling by regulating IRAK degradation (17). DUSP14 inhibits IL-1β–triggered signaling by dephosphorylating TAK1 (18). RBCK1 ubiquitinates and down-regulates TAB2/3 to negatively regulate IL-1β–induced NF-κB activation (19). TRIM38 inhibits IL-1β–triggered signaling by mediating lysosome-dependent degradation of TAB2/3 (20). The existence of a wide range of negative regulators emphasizes the need for tight control of IL-1β–triggered inflammatory response, and it is of great interest to identify additional molecules that regulate IL-1β signaling.

Membrane-associated RING-CH 3 (MARCH3) is a member of the MARCH ubiquitin ligase family. The majority of 11 mammalian MARCH proteins share a similar structure, including an N-terminal C4HC3-type RING finger (RING-CH finger) and two or more C-terminal transmembrane (TM) spans (21). Several members of this family have been shown to ubiquitinate and down-regulate transmembrane proteins, such as IL-1RAcP, MHC-I, and ICAM-1 (16, 22, 23). In this study, we identified MARCH3 as a critical negative regulator of IL-1β–triggered signaling and inflammatory response. MARCH3 mediated K48-linked polyubiquitination of IL-1RI at lysine 409 and its lysosomal degradation. Our findings reveal a mechanism for how IL-1β–triggered inflammatory response is attenuated by MARCH3-mediated down-regulation of IL-1β receptor.

Results

Identification of MARCH3 as a Negative Regulator of IL-1β Signaling. Previously, we have demonstrated that MARCH8 negatively regulates IL-1β–triggered signaling by targeting IL-1RAcP (16).

Significance

Infection of pathogenic microbes induces the body to produce cytokines, which are mediators of inflammation. IL-1β plays central roles in the initiation of inflammatory and immune responses. In this study, we identified MARCH3 protein, which negatively regulates IL-1β–triggered signaling and inflammatory response by mediating K48-linked polyubiquitination of IL-1RI at lysine 409 and its lysosomal degradation. Our findings reveal a mechanism for how IL-1β–triggered inflammatory response is attenuated by down-regulation of IL-1β receptor.

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We further screened eight MARCH family members for their effects on IL-1β–triggered signaling and identified MARCH3 as a candidate (*SI Appendix*, Fig. S1). Overexpression of MARCH3 inhibited IL-1β–induced NF-κB activation in a dose-dependent manner (Fig. 1*A*). The E3 ligase inactive mutants of MARCH3, including C71S, C74S, and C87S, had no marked inhibitory effects on IL-1β–induced NF-κB activation (Fig. 1*B*). MARCH3 had no marked effects on NF-κB activation triggered by IL-33, a similar agonistic member of the IL-1 family (Fig. 1*C*). Consistently, overexpression of MARCH3 inhibited IL-1β– but not IL-33–induced transcription of *TNFA*, *IKBA*, *IL8*, and *CXCL1* genes (Fig. 1*D*), and also inhibited IL-1β–induced phosphorylation of IκBα and p65 (*SI Appendix*, Fig. S2*A*).

To determine whether endogenous MARCH3 regulates IL-1β–triggered signaling, we constructed two human MARCH3-RNAi plasmids which could down-regulate the expression of MARCH3 in cells (Fig. 1*E*). Knockdown of MARCH3 potentiated IL-1β– but not IL-33–triggered NF-κB activation (Fig. 1*F*), as well as IL-1β– but not IL-33–induced transcription of *TNFA*, *IKBA*, *IL8*, and *CXCL1* genes in HCT116 cells (Fig. 1*G*). Consistently, knockdown of MARCH3 enhanced IL-1β–induced phosphorylation of IκBα and p65 (*SI Appendix*, Fig. S2*B*). Taken together, these data suggest that MARCH3 specifically inhibits IL-1β– but not IL-33–induced signaling.

March3 Deficiency Potentiates IL-1β–Induced Signaling and Inflammation. To further investigate the functions of MARCH3 in vivo, we generated March3-deficient mice by the CRISPR/Cas9 system (*SI Appendix*, Fig. S3 *A* and *B*). Homozygous *March3*$^{-/-}$ mice were born at the Mendelian ratio and did not display any developmental abnormality, suggesting that March3 is dispensable for the survival and development of mice. qPCR analysis confirmed that *March3*$^{-/-}$ cells were defective at producing *March3* mRNA (*SI Appendix*, Fig. S3*C*).

To investigate the roles of March3 in IL-1β–induced inflammation response, we examined the expression of downstream genes induced by IL-1β in wild-type and *March3*$^{-/-}$ bone marrow-derived macrophages (BMDMs), bone marrow-derived monocytes, and primary mouse lung fibroblasts (MLFs). We found that IL-1β– but not IL-33–induced transcription of *Tnfa* and *Ikba* genes was potentiated in *March3*$^{-/-}$ BMDMs compared with their wild-type counterparts (Fig. 2*A*). Consistently, March3 deficiency potentiated IL-1β–induced transcription of *Tnfa*, *Il1b*, and *Ikba* genes in monocytes and MLFs (*SI Appendix*, Fig. S4). In addition, IL-1β–induced phosphorylation of IκBα and p65 was increased in *March3*$^{-/-}$ in comparison with wild-type BMDMs (Fig. 2*B*). These results suggest that March3 deficiency potentiates IL-1β–induced signaling in murine cells.

To investigate whether March3 regulates IL-1β–induced inflammatory response in vivo, we examined IL-1β–induced production of proinflammatory cytokines in the sera of mice. Age- and sex-matched wild-type and March3-deficient mice were injected intraperitoneally (i.p.) with murine IL-1β. We found that serum cytokines induced by IL-1β, including TNFα and IL-1β, were markedly increased in *March3*$^{-/-}$ in comparison with the wild-type mice (Fig. 2*C*). Consistently, after injection with IL-1β plus D-galactosamine, *March3*$^{-/-}$ mice experienced early death onset and a higher percentage of lethality within 30 h compared with their wild-type counterparts (Fig. 2*D*). These data suggest that March3 plays an important role in negative regulation of IL-1β–triggered inflammatory response in vivo.

March3 Deficiency Potentiates Inflammatory Response to *Listeria monocytogenes*. It has been shown that IL-1β plays an important role in inflammatory response to infection of bacteria such as *Listeria monocytogenes* (24). We therefore determined whether March3 plays a role in inflammatory response to *L. monocytogenes*. As shown in Fig. 3*A*, *March3*$^{-/-}$ mice carried less *L. monocytogenes* in their spleens and livers compared with that of their wild-type littermates at 2 d post-i.p. of *L. monocytogenes*, suggesting that *March3*$^{-/-}$ mice exhibited more efficient clearance of invaded *L. monocytogenes* than the wild-type mice. *March3*$^{-/-}$ mice produced much higher levels of inflammatory cytokines, including TNF-α and IL-1β (Fig. 3*B*) and showed much more serious inflammatory damage of their lungs (Fig. 3*C*) and small intestinal villus (Fig. 3*D*) after *L. monocytogenes* infection by i.p., which led to a higher sensitivity to *L. monocytogenes*-induced inflammatory death of *March3*$^{-/-}$ mice (Fig. 3*E*). Anakinra treatment rendered both

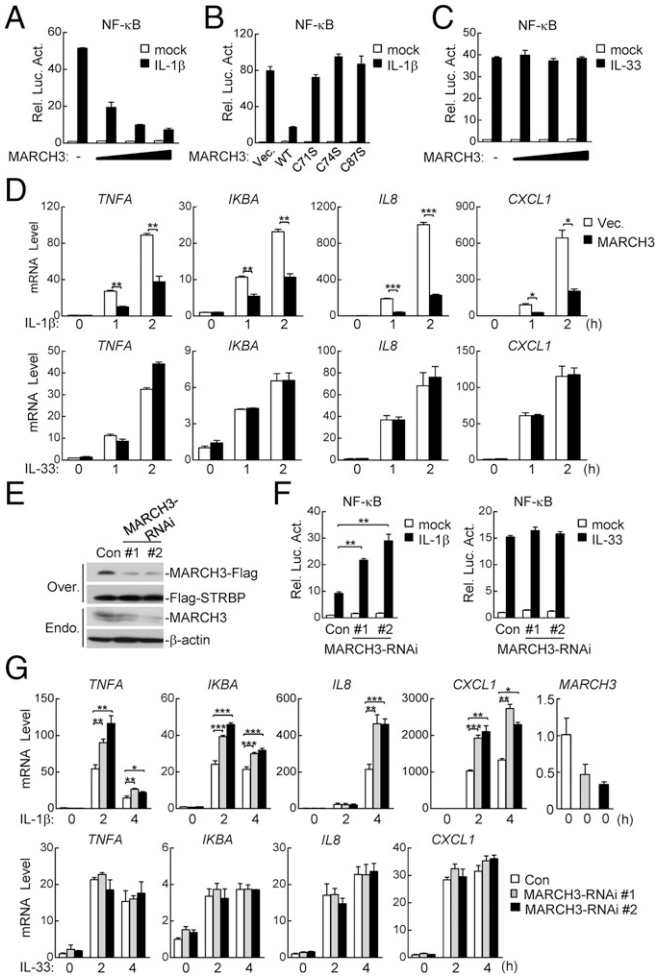

Fig. 1. MARCH3 inhibits IL-1β–triggered signaling. (*A*) Effects of MARCH3 on IL-1β–triggered NF-κB activation. HEK293 cells were transfected with NF-κB reporter (10 ng) and MARCH3 plasmid (25, 50, and 100 ng) for 24 h. The cells were then treated with IL-1β (10 ng/mL) or left untreated for 10 h before reporter assays. (*B*) Effects of MARCH3 mutants on IL-1β–triggered NF-κB activation. HEK293 cells were transfected with NF-κB reporter (10 ng) and MARCH3 or its mutants for 24 h. The cells were then treated with IL-1β (10 ng/mL) or left untreated for 10 h before reporter assays. (*C*) Effects of MARCH3 on IL-33–induced NF-κB activation. The experiments were similarly performed as in *A* except that ST2 was used to replace IL-1RI for transfection, and IL-33 (20 ng/mL) was used to treat cells. (*D*) Effects of MARCH3 on IL-1β– or IL-33–induced transcription of inflammation genes. HCT116 cells were transduced with either an empty vector or MARCH3-Flag plasmid to establish stable cell lines. The cells were treated with IL-1β (10 ng/mL) or IL-33 (100 ng/mL) for the indicated times before qPCR experiments. (*E*) Effects of MARCH3-RNAi on expression of MARCH3. *Upper* shows HEK293 cells were transfected with expression plasmids for MARCH3-Flag and Flag-STRBP, and the indicated MARCH3-RNAi plasmids for 24 h before immunoblot with anti-Flag. *Lower* shows HEK293 cells were transfected with MARCH3-RNAi plasmids for 48 h before immunoblotting analysis. (*F*) Effects of MARCH3 knockdown on IL-1β– and IL-33–induced NF-κB activation. HEK293 cells were transfected with empty vector or ST2, MARCH3-RNAi, and NF-κB reporter plasmids as indicated for 36 h, then left untreated or treated with IL-1β (10 ng/mL) or IL-33 (20 ng/mL) for 10 h before reporter assays. (*G*) Effects of MARCH3 knockdown on IL-1β– or IL-33–induced transcription of inflammatory genes. HCT116 cells stably transduced with control or MARCH3-RNAi were treated with IL-1β (10 ng/mL) or IL-33 (100 ng/mL) for the indicated times before qPCR experiments. Data are mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

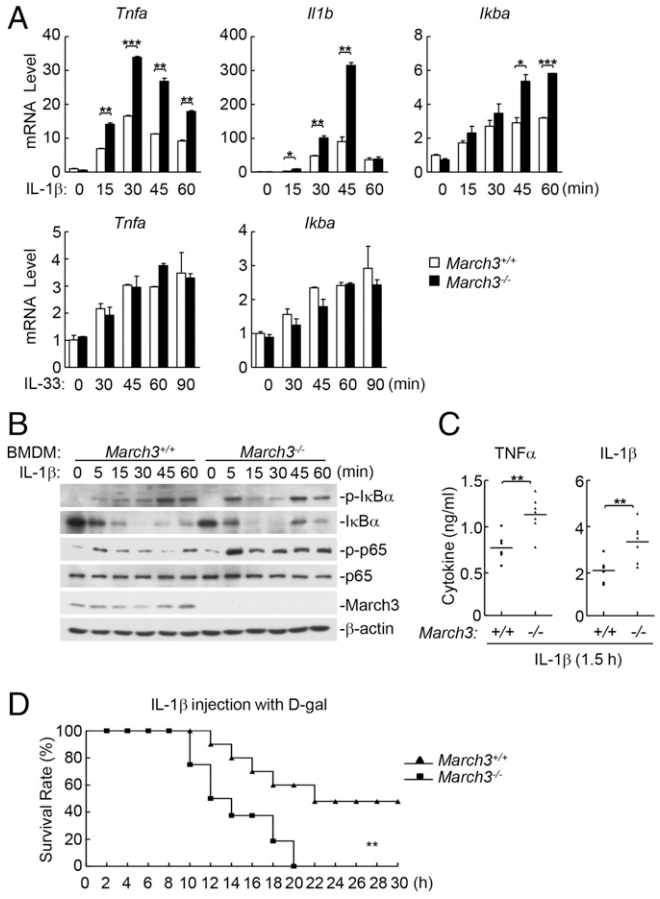

Fig. 2. March3 deficiency potentiates IL-1β–induced signaling and inflammation. (*A*) Effects of March3 deficiency on IL-1β– or IL-33–induced transcription of inflammatory genes. *March3*$^{+/+}$ and *March3*$^{-/-}$ BMDMs were left untreated or treated with murine IL-1β (50 ng/mL) or IL-33 (50 ng/mL) for the indicated times before qPCR experiments. Graphs are mean ± SD ($n = 3$). (*B*) Effects of March3 deficiency on IL-1β–induced phosphorylation of IκBα and p65. *March3*$^{+/+}$ and *March3*$^{-/-}$ BMDMs were left untreated or treated with murine IL-1β (100 ng/mL) for the indicated times before immunoblotting analysis. (*C*) Effects of March3 deficiency on IL-1β–induced serum cytokine levels. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ mice ($n = 7$, 8 wk old) were injected i.p. with murine IL-1β (150 μg/kg) for the indicated times followed by measurement of the indicated serum cytokines by ELISA. (*D*) Effects of March3 deficiency on IL-1β–induced inflammatory death. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ mice ($n = 7$, 8 wk old) were injected i.p. with murine IL-1β (150 μg/kg) plus D-galactosamine (1 mg/g) per mouse. The survival curve was generated by Kaplan–Meier methods followed by log-rank test analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

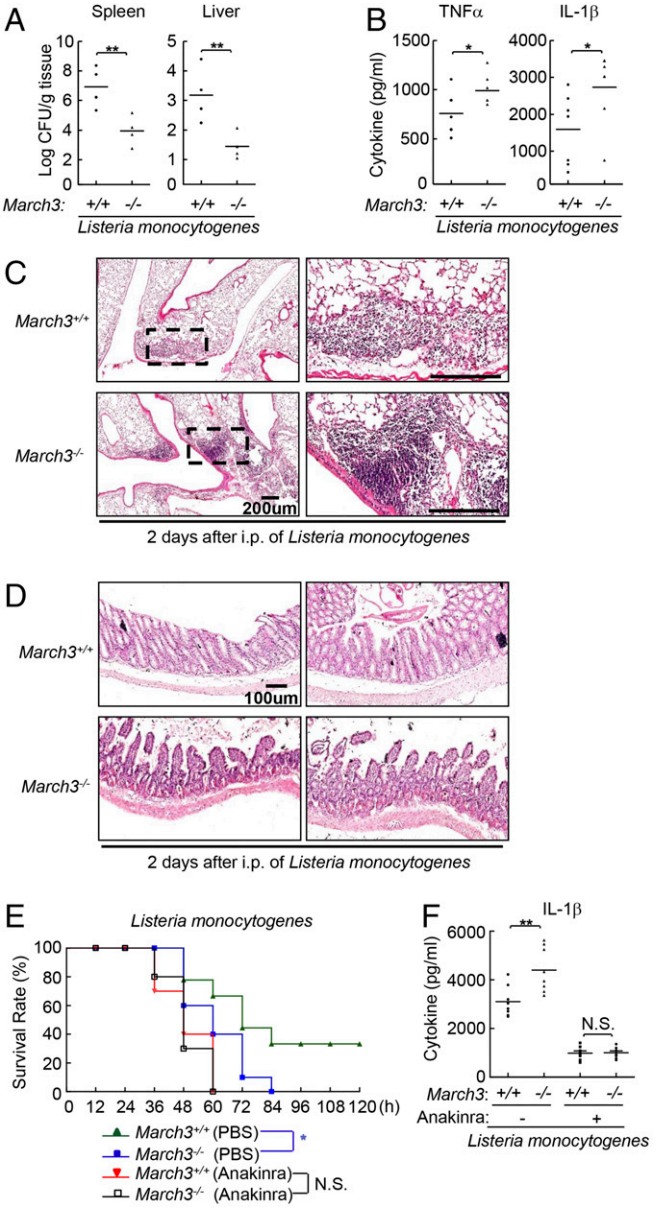

Fig. 3. March3 deficiency potentiates inflammatory response to *L. monocytogenes*. (*A*) Effects of March3 deficiency on clearance of *L. monocytogenes* in mice. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ mice ($n = 4$) were i.p. infected with *L. monocytogenes*, and bacterial loads were assessed in the indicated tissues 2 d postinfection. (*B*) Effects of March3 deficiency on *Listeria*-induced secretion of inflammatory cytokines. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ mice ($n = 7$) were i.p. infected with *L. monocytogenes* for 12 h, followed by measurement of the indicated serum cytokine levels by ELISA. (*C* and *D*) Effects of March3 deficiency on *Listeria*-induced inflammatory damage of the tissues. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ mice were i.p. infected with *L. monocytogenes*, and the lungs (*C*) or intestines (*D*) of mice were analyzed by histology. (*E*) Effects of March3 deficiency on *Listeria*-induced inflammatory death. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ ($n = 10$) mice were injected i.v. with PBS or anakinra (20 ng/g) for 2 h and then infected (i.p.) with *L. monocytogenes*. The survival rates of mice were observed daily. (*F*) Effects of anakinra on *Listeria*-induced secretion of cytokines. Sex- and age-matched *March3*$^{+/+}$ and *March3*$^{-/-}$ ($n = 8$) mice were injected i.v. with PBS or anakinra (20 ng/g) for 2 h and then infected (i.p.) with *L. monocytogenes* for 24 h before measurement of the indicated serum cytokines by ELISA. NS, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$.

the wild-type and March3-deficient mice more susceptible to *L. monocytogenes*-induced death, and abolished the difference between wild-type and *March3*$^{-/-}$ mice in *L. monocytogenes*-induced inflammatory death or inflammatory cytokine production (Fig. 3 *E* and *F*). These results suggest that IL-1 activity is important for protecting mice from *L. monocytogene*-induced death, but March3 can act to block *L. monocytogenes*-induced cytokine "storm" and death.

MARCH3 Mediates Lysosomal-Dependent Degradation of IL-1RI. To investigate the molecular mechanisms for how MARCH3 regulates IL-1β–triggered signaling, we determined effects of MARCH3 on NF-κB activation mediated by key components of the IL-1R–mediated pathways. In reporter assays, MARCH3 inhibited NF-κB activation mediated by overexpression of IL-1RI/IL-1RAcP but not MyD88, IRAK1, TRAF6, TAK1/TAB1, TAB2, TAB3, or IKKβ. In these experiments, MARCH3 did not inhibit NF-κB activation triggered by the IL-33 receptor complex

ST2/IL-1RAcP (*SI Appendix*, Fig. S5*A*). The protein level of IL-1RI/IL-1RAcP was down-regulated when coexpressed with MARCH3 (*SI Appendix*, Fig. S5*A*). In addition, the E3 ligase-inactive mutants of MARCH3, C71S, C74S, and C87S, did not inhibit NF-κB activation mediated by IL-1RI/IL-1RAcP (*SI Appendix*, Fig. S5*A*). These results suggest that MARCH3 targets IL-1 receptor complex in a ligase activity-dependent manner.

In transient transfection and coimmunoprecipitation experiments, MARCH3 interacted with IL-1RI and IL-1RAcP but not MyD88 (Fig. 4*A*). Endogenous coimmunoprecipitation experiments showed that MARCH3 constitutively interacted with IL-1RI before and after IL-1β stimulation in a dynamic manner in HCT116 cells: their association was decreased at 30 min and then further increased at 60 min after IL-1β stimulation (Fig. 4*B*). Domain mapping experiments indicated that the TM and TIR domains of IL-1RI are required and sufficient for their interaction with MARCH3 (*SI Appendix*, Fig. S5*B*), while the second TM of MARCH3 was important for its interaction with IL-1RI (*SI Appendix*, Fig. S5*C*).

Interestingly, we found that the protein level of IL-1RI, but not ST2, IL-1RAcP, or MyD88 was dramatically down-regulated when coexpressed with MARCH3 (Fig. 4*C*). In similar experiments, MARCH3 C71S, C74S, and C87S did not down-regulate the levels of IL-1RI (*SI Appendix*, Fig. S5*D*). These results suggest that MARCH3 specially mediates degradation of IL-1RI in an E3 ligase activity-dependent manner. Previously, it has been reported that IL-1β binding causes IL-1RI internalization and subsequent lysosome-dependent degradation (25). We found that knockdown of MARCH3 up-regulated the level of endogenous IL-1RI and reversed IL-1β–induced degradation of IL-1RI (Fig. 4*D*). Furthermore, we found that MARCH3-mediated down-regulation of IL-1RI was restored by NH_4Cl (Fig. 4*E*), which is an inhibitor of lysosome-dependent degradation. Knockdown of LAMP2, which is a key component of lysosome-dependent

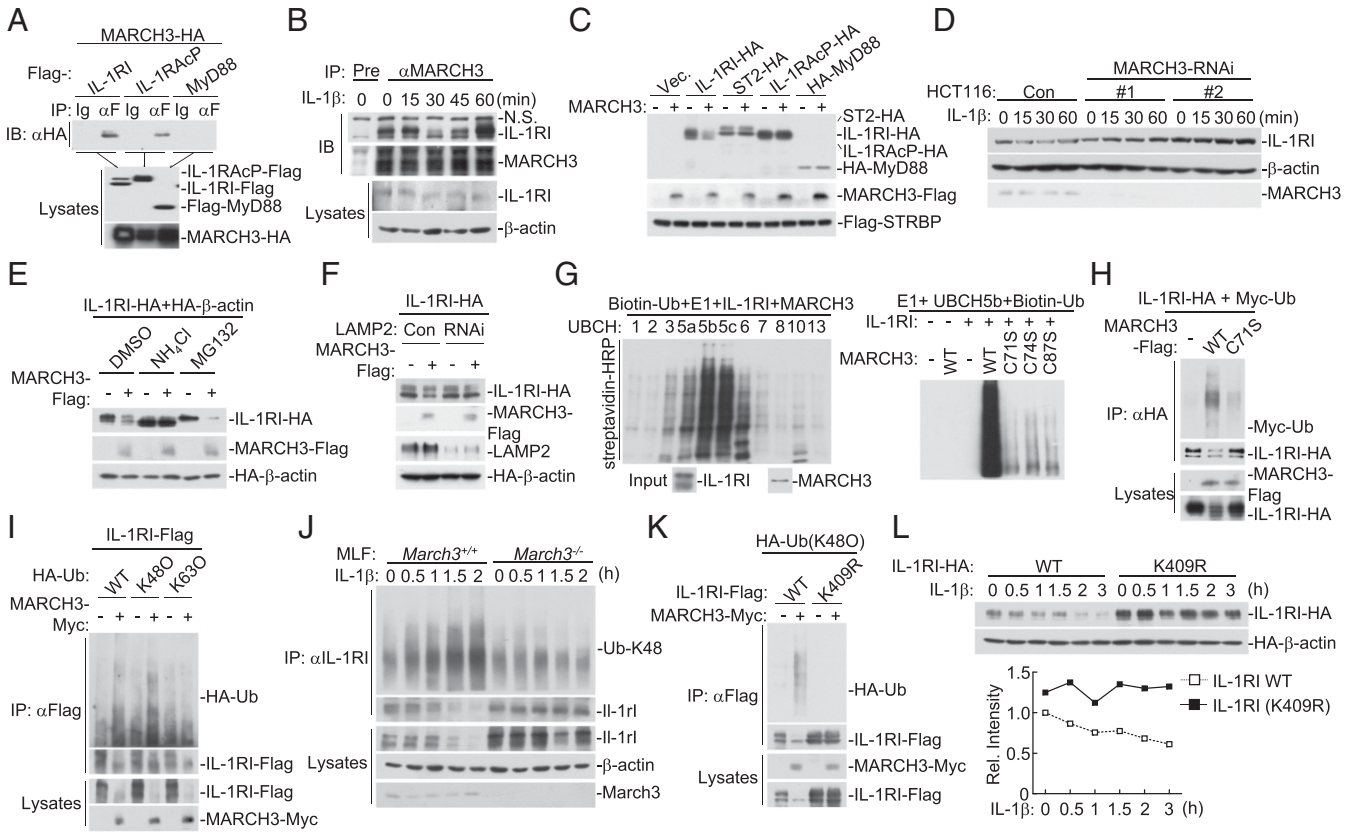

Fig. 4. MARCH3 mediates K48-linked polyubiquitination of IL-1RI at K409. (*A*) MARCH3 interacts with IL-1RI. HEK293 cells were transfected with the indicated plasmids for 20 h before coimmunoprecipitation and immunoblotting analysis. (*B*) Endogenous MARCH3 is associated with IL-1RI. HCT116 cells were left untreated or treated with IL-1β (100 ng/mL) for the indicated times before coimmunoprecipitation and immunoblotting analysis. N.S., not significant. (*C*) MARCH3 promotes degradation of IL-1RI. HEK293 cells were transfected with the indicated plasmids for 24 h before immunoblotting analysis. (*D*) Effects of MARCH3 knockdown on IL-1β–induced degradation of IL-1RI. HCT116 cells stably transduced with control or MARCH3-RNAi were treated with IL-1β (50 ng/mL) for the indicated times before immunoblotting analysis. (*E*) Effects of inhibitors on IL-1RI levels. HEK293 cells were transfected with the indicated plasmids for 10 h and then NH_4Cl (50 μmol/mL) or MG132 (50 nmol/mL) were added to the medium for 6 h before immunoblotting analysis. (*F*) Effects of LAMP2 knockdown on MARCH3-mediated degradation of IL-1RI. HEK293 cells were transfected with LAMP2-RNAi plasmids for 24 h. Then the indicated plasmids were transfected for 20 h before immunoblotting analysis. (*G*) MARCH3 but not its mutants mediates polyubiquitination of IL-1RI in vitro. IL-1RI, MARCH3, and MARCH3 (C71S, C74S, and C87S) were translated in vitro, and then E1, the indicated E2s, and biotin-ubiquitin were added for in vitro ubiquitination assays. The ubiquitin-conjugated proteins were detected by immunoblots with streptavidin-HRP. The expression levels of the related proteins were examined by immunoblots with the indicated antibodies. (*H*) MARCH3 but not its C71S mutant mediates polyubiquitination of IL-1RI in cells. HEK293 cells were transfected with the indicated plasmids for 20 h. Ubiquitination assays were performed with the indicated antibodies. (*I*) MARCH3 mediates K48-linked polyubiquitination of IL-1RI. HEK293 cells were transfected with the indicated plasmids for 20 h before ubiquitination assays. (*J*) Effects of March3 deficiency on IL-1β–induced K48-linked polyubiquitination and degradation of IL-1RI. *March3*$^{+/+}$ and *March3*$^{-/-}$ MLFs were left untreated or treated with murine IL-1β (400 ng/mL) for the indicated times. The cell lysates were immunoprecipitated with mouse anti–IL-1RI, and the immunoprecipitates were analyzed by immunoblots with anti-Ub (K48-specific) or rabbit anti–IL-1RI. (*K*) MARCH3 does not mediate K48-linked polyubiquitination of IL-1RI(K409R). HEK293 cells were transfected with the indicated plasmids for 24 h before ubiquitination assays. (*L*) Resistance of IL-1RI(K409R) to IL-1β–induced degradation. HEK293 cells were transfected with the indicated plasmids for 15 h and then treated with cycloheximide (CHX) (250 μg/mL) for 1 h. Cells then were left untreated or treated with IL-1β (100 ng/mL) for the indicated times before immunoblotting analysis.

degradation complex (26), inhibited MARCH3-mediated down-regulation of IL-1RI (Fig. 4*F*). Taken together, these data suggest that MARCH3 specially mediates lysosomal-dependent degradation of IL-1RI.

MARCH3 Mediates K48-Linked Polyubiquitination of IL-1RI. Since MARCH3 is an E3 ubiquitin ligase and mediates degradation of IL-1RI, we determined whether MARCH3 mediates polyubiquitination of IL-1RI. In vitro ubiquitination assays with recombinant proteins indicated that MARCH3 catalyzed polyubiquitination of IL-1RI with UBCH5b or UBCH5c as an E2, and the MARCH3 C71S, C74S, and C87S mutant had little activity in these assays (Fig. 4*G*). In the mammalian overexpression system, MARCH3 but not its C71S mutant markedly enhanced polyubiquitination of IL-1RI (Fig. 4*H*). Further experiments with linkage-specific ubiquitin indicated that MARCH3 promoted K48-linked but not K63-linked polyubiquitination of IL-1RI (Fig. 4*I*). Endogenous ubiquitination assays indicated that IL-1β stimulation increased K48-linked polyubiquitination and degradation of IL-1RI in wild-type MLFs, and these effects were reversed in March3-deficient MLFs (Fig. 4*J*). Collectively, these results suggest that MARCH3 directly mediates K48-linked polyubiquitination of IL-1RI to facilitate its degradation after IL-1β stimulation.

MARCH3 Ubiquitinates IL-1RI at Lysine 409. To identify potential ubiquitination residues of IL-1RI, we first mapped the region targeted by MARCH3. As shown in *SI Appendix*, Fig. S6*A*, MARCH3 mediated polyubiquitination of the C-terminal (amino acids 334–569) but not the N-terminal (amino acids 1–336) region of IL-1RI in the mammalian overexpression system. We mutated all of the 21 lysine residues within amino acids 334–569 to arginine individually and examined whether NF-κB activation by these mutants could be inhibited by MARCH3. As shown in *SI Appendix*, Fig. S6*B*, MARCH3 inhibited NF-κB activation mediated by all mutants except IL-1RI(K409R). Consistently, the protein levels of all mutants except IL-1RI (K409R) were markedly down-regulated by MARCH3 (*SI Appendix*, Fig. S6*C*). Interestingly, sequence analysis showed that K409 of IL-1RI was conserved in various species (*SI Appendix*, Fig. S6*D*), suggesting that K409 of IL-1RI has a conserved role. Consistently, MARCH3 mediated polyubiquitination of wild-type IL-1RI but not IL-1RI(K409R) (Fig. 4*K*). Moreover, mutation of K409 to arginine markedly inhibited IL-1β–induced degradation of IL-1RI (Fig. 4*L*). Taken together, these results suggest that MARCH3 mediates IL-1β–induced polyubiquitination at K409 and degradation of IL-1RI.

CDC25A Dephosphorylates Tyrosine Phosphorylation of MARCH3. Our earlier experiments indicated that MARCH3 was constitutively associated with IL-1RI in unstimulated cells, but K48-linked polyubiquitination and degradation of IL-1RI occurred after IL-1β stimulation. We reasoned that MARCH3 was activated following IL-1β stimulation. We next investigated whether IL-1β stimulation activates MARCH3 by phosphorylation and dephosphorylation. As shown in Fig. 5*A*, endogenous MARCH3 was modified by tyrosine phosphorylation in unstimulated cells, which was decreased after IL-1β stimulation. To identify the protein tyrosine phosphatases (PTPs) responsible for IL-1β–triggered dephosphorylation, we screened 39 tyrosine phosphatases for their effects on IL-1β–triggered signaling. As shown in *SI Appendix*, Fig. S7*A*, overexpression of CDC25A, CDC25B, and DUSP16 inhibited IL-1β–induced NF-κB activation. In transient transfection and coimmunoprecipitation experiments, CDC25A but not CDC25B or DUSP16 interacted with MARCH3 (*SI Appendix*, Fig. S7*B*). Knockdown of CDC25A potentiated IL-1β–induced NF-κB activation (*SI Appendix*, Fig. S7 *C* and *D*), as well as transcription of *TNFA*, *CXCL1*, and *IKBA* genes in HCT116 cells (*SI Appendix*, Fig. S7*E*). Consistently, knockdown of CDC25A increased IL-1β–induced tyrosine phosphorylation of endogenous MARCH3 (Fig. 5*B*). Endogenous ubiquitination assays indicated that IL-1β stimulation increased K48-linked polyubiquitination and degradation of IL-1RI in HCT116 cells, while these effects were reversed in CDC25A-knockdown cells (Fig. 5*C*). Collectively, these results suggest that CDC25A dephosphorylates MARCH3 to activate its E3 ligase activity after IL-1β stimulation.

TYRO3 Mediates Tyrosine Phosphorylation of MARCH3. We next attempted to identify the protein tyrosine kinases (PTKs) that mediate phosphorylation of MARCH3. We screened 42 PTK family members for their effects on IL-1β–triggered signaling.

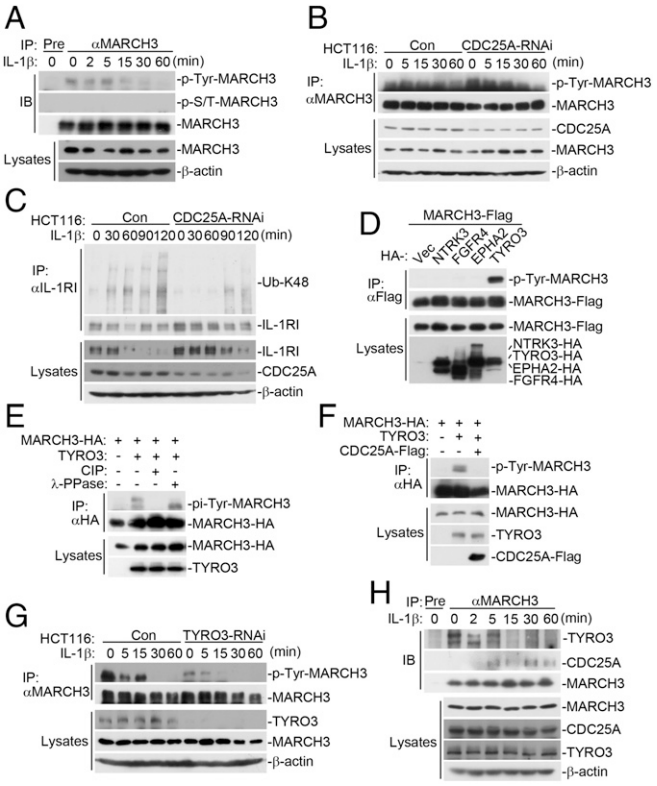

Fig. 5. CDC25A dephosphorylates TYRO3-mediated tyrosine phosphorylation of MARCH3. (*A*) IL-1β attenuated tyrosine phosphorylation of MARCH3. HCT116 cells were left untreated or treated with IL-1β (100 ng/mL) for the indicated times before coimmunoprecipitation and immunoblotting analysis. (*B*) Effects of CDC25A knockdown on IL-1β–induced tyrosine phosphorylation of MARCH3. HCT116 cells stably transduced with control or CDC25A-RNAi were treated with IL-1β (100 ng/mL) for the indicated times before coimunoprecipitation and immunoblotting analysis. (*C*) Effects of CDC25A knockdown on IL-1β–induced K48-linked polyubiquitination and degradation of IL-1RI. HCT116 cells stably transduced with control or CDC25A-RNAi were treated with IL-1β (100 ng/mL) for the indicated times before coimunoprecipitation and immunoblotting analysis. (*D*) Effects of tyrosine kinases on phosphorylation of MARCH3. HEK293 cells were transfected with the indicated plasmids for 20 h before coimmunoprecipitation and immunoblotting analysis. (*E*) Effects of CIP on TYRO3-mediated tyrosine phosphorylation of MARCH3. HEK293 cells were transfected with the indicated plasmids for 20 h. Cell lysates were immunoprecipitated with anti-HA, and the immunoprecipitates were treated with CIP before immunoblotting analysis. (*F*) Effects of CDC25A on TYRO3-mediated tyrosine phosphorylation of MARCH3. HEK293 cells were transfected with the indicated plasmids for 20 h before coimmunoprecipitation and immunoblotting analysis. (*G*) Effects of TYRO3 knockdown on IL-1β–induced tyrosine phosphorylation of MARCH3. HCT116 cells stably transduced with control or TYRO3-RNAi were treated with IL-1β (100 ng/mL) for the indicated times before coimmunoprecipitation and immunoblotting analysis. (*H*) Endogenous MARCH3 is associated with TYRO3 and CDC25A. HCT116 cells were left untreated or treated with IL-1β (100 ng/mL) for the indicated times before coimmunoprecipitation and immunoblotting analysis.

We found that overexpression of TYRO3, NTRK3, FGFR4, and EPHA2 potentiated IL-1β–induced NF-κB activation when cotransfected with MARCH3 (*SI Appendix*, Fig. S8*A*). Furthermore, overexpression of TYRO3 but not NTRK3, FGFR4, and EPHA2 caused tyrosine phosphorylation of MARCH3 (Fig. 5*D*), which could be removed by treatment with calf intestinal alkaline phosphatase (CIP) (Fig. 5*E*). Consistently, CDC25A could dephosphorylate TYRO3-mediated tyrosine phosphorylation of MARCH3 (Fig. 5*F*). Knockdown of TYRO3 inhibited IL-1β–triggered NF-κB activation (*SI Appendix*, Fig. S8 *B* and *C*), transcription of *TNFA*, *CXCL1*, and *IKBA* genes (*SI Appendix*, Fig. S8*D*), and tyrosine phosphorylation of endogenous MARCH3 (Fig. 5*G*). Endogenous coimmunoprecipitation experiments showed that TYRO3 was constitutively associated with MARCH3 in unstimulated cells and this association was decreased following IL-1β stimulation. In contrast, CDC25A was associated with MARCH3 in an IL-1β stimulation-dependent manner in HCT116 cells (Fig. 5*H*). Taken together, these results suggest that MARCH3 is kept inactive by TYRO3-mediated phosphorylation in unstimulated cells, and dephosphorylated by CDC25A to promote its activation and K48-linked polyubiquitination of IL-1RI, leading to inhibition of IL-1RI–mediated signaling.

Discussion

IL-1β is an important cytokine critically involved in inflammatory response. IL-1β–triggered signaling is tightly regulated to avoid host immune damage. In this report, we identified MARCH3 as a critical negative regulator of IL-1β–triggered signaling by targeting IL-1RI for K48-linked polyubiquitination at K409 and lysosomal-dependent degradation.

Overexpression of MARCH3 inhibited IL-1β– but not IL-33–triggered activation of NF-κB and induction of inflammatory genes, whereas MARCH3 deficiency had opposite effects, suggesting that MARCH3 specifically regulates IL-1β–triggered signaling. March3 deficiency also increased the serum cytokine levels and inflammatory response induced by IL-1β as well as promoted clearance of *L. monocytogenes* and increased the susceptibility to *L. monocytogenes*-triggered inflammatory death. These results demonstrate that March3 attenuates IL-1β–triggered inflammatory response in vivo.

Our studies suggest that MARCH3 negatively regulates IL-1β–triggered signaling by mediating K48-linked polyubiquitination and lysosome-dependent degradation of IL-1RI. Overexpression of MARCH3 caused down-regulation of IL-1RI and inhibited IL-1RI–mediated NF-κB activation in an E3 ubiquitin ligase activity-dependent manner. MARCH3 promoted K48-linked polyubiquitination of IL-1RI at lysine 409 after IL-1β stimulation, which is important for its degradation. Moreover, MARCH3-mediated degradation of IL-1RI was inhibited by the lysosomal inhibitor NH_4Cl, as well as by deficiency of the lysosomal degradation complex member LAMP2.

Our studies also suggest that the E3 ligase activity of MARCH3 is induced after IL-1β stimulation. Our results indicated that MARCH3 was kept inactive by TYRO3-mediated phosphorylation in unstimulated cells. Upon IL-1β stimulation, CDC25A could dephosphorylate MARCH3, which in turn activated MARCH3 and caused K48-linked polyubiquitination and degradation of IL-1RI, leading to inhibition of IL-1RI–mediated signaling.

Previously, it has been reported that MARCH8, another member of the MARCH family, promotes K48-linked ubiquitination and proteasome-dependent degradation of IL-1RAcP but not IL-1RI (16). In contrast, we found that MARCH3 catalyzed IL-1β–induced K48-linked polyubiquitination and lysosome-dependent degradation of IL-1RI but not IL-1RAcP. Furthermore, MARCH3 had no effects on IL-33–induced signaling and degradation of the IL-33 receptor ST2. These observations suggest that different components of the IL-1 receptor complex as well as specific receptors for the IL-1 family of ligands are versatilely regulated by distinct members of the MARCH family, which provides a layer of precise control of inflammatory and immunological responses.

Materials and Methods

All animal experiments were performed in accordance with the Wuhan University Animal Care and Use Committee guidelines. The information on reagents, antibodies, cells, constructs, PCR primers, and RNAi target sequences are described in *SI Appendix, SI Materials and Methods*. The methods for generation of bone marrow-derived monocytes and macrophages, isolation of MLFs, cell lines, and retroviral gene transfer, transfection, reporter assays, coimmunoprecipitation, immunoblot analysis, and statistical analysis are previously described (27, 28) and the details are presented in *SI Appendix, SI Materials and Methods*.

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