UAF1 deubiquitinase complexes facilitate NLRP3 inflammasome activation by promoting NLRP3 expression

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NOD-like receptor protein 3 (NLRP3) detects microbial infections or endogenous danger signals and activates the NLRP3 inflammasome, which has important functions in host defense and contributes to the pathogenesis of inflammatory diseases, and thereby needs to be tightly controlled. Deubiquitination of NLRP3 is considered a key step in NLRP3 inflammasome activation. However, the mechanisms by which deubiquitination controls NLRP3 inflammasome activation are unclear. Here, we show that the UAF1/USP1 deubiquitinase complex selectively removes K48-linked polyubiquitination of NLRP3 and suppresses its ubiquitination-mediated degradation, enhancing cellular NLRP3 levels, which are indispensable for subsequent NLRP3 inflammasome assembly and activation. In addition, the UAF1/USP12 and UAF1/USP46 complexes promote NF-κB activation, enhance the transcription of NLRP3 and proinflammatory cytokines (including pro-IL-1β, TNF, and IL-6) by inhibiting ubiquitination-mediated degradation of p65. Consequently, Uaf1 deficiency attenuates NLRP3 inflammasome activation and IL-1β secretion both in vitro and in vivo. Our study reveals that the UAF1 deubiquitinase complexes enhance NLRP3 and pro-IL-1β expression by targeting NLRP3 and p65 and licensing NLRP3 inflammasome activation.
The NLRP3 inflammasome is a multifunctional complex, comprising a NOD-like receptor NLRP3, an adapter protein apoptosis-associated speck-like protein (ASC), and an effector pro-caspase-1, and plays fundamental roles in inflammation1–4. The NLRP3 inflammasome recognizes pathogen-associated molecular patterns (PAMPs) from invading microbes and endogenous danger signals (damage-associated molecular patterns, DAMPs) released from damaged or dying cells4–8. NLRP3 assembles an inflammasome complex with ASC and pro-caspase-1 after receiving a priming signal (signal 1) from toll-like receptors (TLRs) and an activation signal (signal 2) from respective NLRP3 inflammasome activators, such as extracellular ATP, nigericin (Nig), alum, crystals, amyloid-β, and others. The NLRP3 inflammasome complex subsequently serves as a platform for self-clearance and activation of the cysteine protease caspase-1, promotes the maturation and secretion of IL-1β and IL-18, and induces pyroptosis. Aberrant NLRP3 inflammasome activation is involved in many types of diseases, such as infectious diseases, gout, type 2 diabetes, atherosclerosis, Alzheimer’s disease, and cancers. Therefore, NLRP3 inflammasome activity should be tightly regulated to avoid such detrimental disorders.

Ubiquitination is a key post-translational modification (PTM) that controls NLRP3 inflammasome activation. In resting macrophages, NLRP3 is polyubiquitinated with mixed K48 and K63 ubiquitin chains, which is crucial for the maintenance of NLRP3 inactivation. NLRP3 is deubiquitytated upon priming and activation, and that is a key step in the formation and activation of the NLRP3 inflammasome. ABRO1 recruits BRCC3 to remove K63-linked ubiquitination of NLRP3, facilitating the assembly and activation of NLRP3 inflammasome. K48-linked ubiquitination mediates protein degradation of NLRP3 and thus limits NLRP3 inflammasome activation. Although several E3 ubiquitin ligases such as TRIM31, March7, ARH12, and FBXL29,12–14 have been reported to attenuate NLRP3 inflammasome activation by mediating NLRP3 protein degradation, the function of K48-linked deubiquitination on NLRP3 inflammasome activity remains largely unclear. Whether any deubiquitinating enzymes exist to specifically remove K48-linked ubiquitination of NLRP3, stabilize its expression, and thus license NLRP3 inflammasome activation, remains to be investigated.

Ubiquitin-specific peptidase 1 (USP1)-associated factor 1 (UAF1, also called WDR48 or p80) is a stoichiometric binding partner of three deubiquitinating enzymes and constitutes three deubiquitinating enzyme complexes, including UAF1/USP1, UAF1/USP12, and UAF1/USP46. UAF1 constitutively binds to USP1, USP12, and USP46, and this binding greatly enhances their deubiquitinating activity. The UAF1/USP1 complex deubiquitinates a wide range of substrates and has been implicated in the regulation of DNA repair processes and antiviral innate immunity. USP1 can deubiquitinate and stabilize inhibitors of DNA binding proteins (ID) and subsequent promote the maintenance of mesenchymal stem cells in osteosarcoma. USP12 and USP46 are also involved in tumor pathogenesis by deubiquitinating and stabilizing different targets, such as PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1), TP53 and androgen receptor (AR)24–28. USP12 deubiquitylates and prevents lysosomal degradation of LAT and Trat1 to maintain the proximal T-cell receptor (TCR) complex for the duration of signaling. However, the potential roles of UAF1 deubiquitinase complexes in inflammation are unclear.

Here, we show that UAF1 facilitates NLRP3 inflammasome activation by recruiting the deubiquitinases USP1, USP12, and USP46. The UAF1/USP1 complex interacts with NLRP3, removes its K48-linked polyubiquitination, and stabilizes NLRP3 protein. The UAF1/USP12 and UAF1/USP46 complexes interact with p65 and inhibit its ubiquitination and degradation, thus promoting NF-κB activation, resulting in the enhancements of NLRP3 and pro-IL-1β expressions. Consequently, the UAF1 deubiquitinase complexes facilitates NLRP3 inflammasome activation via targeting NLRP3 and p65. Our study thus uncovers mechanisms regulating NLRP3 inflammasome activation and suggests a promising approach for modulating NLRP3-dependent immunopathologies.

Results

UAF1 facilitates NLRP3 inflammasome activation. To investigate the potential role of UAF1 in NLRP3 inflammasome activation, we first examined the effects of Uaf1 deficiency on IL-1β secretion and caspase-1 cleavage. Systemic deletion of Uaf1 causes early embryonic lethality in mice. Therefore, we generated Uaf1fl/fl mice and then crossed them with Lys2-Cre mice to specifically knockout Uaf1 in myeloid cells (called ‘Uaf1KO’ here) (Supplementary Fig. 1). ATP-stimulated and Nig-stimulated IL-1β secretion and caspase-1 cleavage in LPS-primed Uaf1-deficient mouse peritoneal macrophages were markedly attenuated (Fig. 1a–c). In addition, Uaf1 deficiency could inhibit TNF and IL-6 secretions. To confirm the intrinsic role of Uaf1, siRNAs targeting mouse Uaf1 were used to suppress endogenous UAF1 expression (Supplementary Fig. 2a). Uaf1 knockdown substantially suppressed NLRP3 inflammasome activation-dependent IL-1β secretion and caspase-1 cleavage in mouse peritoneal macrophages (Supplementary Fig. 2b, c). We next investigated the physiological relevance of the effects of UAF1 on NLRP3 inflammasome activation in vivo. Induction of IL-1β by intraperitoneal (i.p.) injection of LPS was shown to be NLRP3-dependent. In our study, IL-1β secretion induced by the LPS injection was much lower in the sera of Uaf1KO mice than in the sera of WT mice, indicating that Uaf1 deficiency inhibits NLRP3 inflammasome activation in vivo (Fig. 1d). Furthermore, TNF and IL-6 secretions were also reduced in the sera of Uaf1KO mice compared with WT mice. Collectively, these results indicate that UAF1 facilitates NLRP3 inflammasome activation, and promotes TNF and IL-6 secretions.

UAF1 enhances NLRP3 expression. Cellular NLRP3 level is vital for the assembly and activation of NLRP3 inflammasome. We then examined the effects of UAF1 on NLRP3 expression. Deficiency and knockdown of Uaf1 markedly inhibited NLRP3 protein expression (Figs. 1b, c and 2a and Supplementary Fig. 2c). In addition, Nlrp3 mRNA expression was also considerably attenuated by Uaf1 deficiency (Fig. 2b). These data indicate that UAF1 selectively controls NLRP3 expression at both the protein and mRNA levels. Uaf1 deficiency had no effect on the expression of AIM2 and NLRC4, other two pattern recognition receptors (PRR), which could form inflammasomes (Fig. 2c). However, IL-1β secretion induced by AIM2 and NLRC4 inflammasome activation was decreased in Uaf1 deficiency (Supplementary Fig. 3a). Furthermore, UAF1 overexpression in NLRP3 and pro-IL-1β expression was shown to be NLRP3-dependent IL-1β secretion and caspase-1 cleavage in LPS-primed Uaf1-deficient mouse peritoneal macrophages were markedly attenuated (Fig. 1a–c). In addition, Uaf1 deficiency could inhibit TNF and IL-6 secretions. To confirm the intrinsic role of Uaf1, siRNAs targeting mouse Uaf1 were used to suppress endogenous UAF1 expression (Supplementary Fig. 2a). Uaf1 knockdown substantially suppressed NLRP3 inflammasome activation-dependent IL-1β secretion and caspase-1 cleavage in mouse peritoneal macrophages (Supplementary Fig. 2b, c). We next investigated the physiological relevance of the effects of UAF1 on NLRP3 inflammasome activation in vivo. Induction of IL-1β by intraperitoneal (i.p.) injection of LPS was shown to be NLRP3-dependent. In our study, IL-1β secretion induced by the LPS injection was much lower in the sera of Uaf1KO mice than in the sera of WT mice, indicating that Uaf1 deficiency inhibits NLRP3 inflammasome activation in vivo (Fig. 1d). Furthermore, TNF and IL-6 secretions were also reduced in the sera of Uaf1KO mice compared with WT mice. Collectively, these results indicate that UAF1 facilitates NLRP3 inflammasome activation, and promotes TNF and IL-6 secretions.

USP1/12/46 enhances NLRP3 expression and inflammasome activation. To investigate whether the regulatory role of UAF1 on
Fig. 1 UAF1 facilitates NLRP3 inflammasome activation. a ELISA analysis of IL-1β, IL-6 and TNF secretion in mouse peritoneal macrophages from wild-type (WT) or Uaf1CKO mice following priming with LPS for 8 h and subsequent stimulation with ATP or Nig for 40 min. US, unstimulated (mean ± SEM, two-tailed t-test Uaf1CKO vs. WT, left panel: **P=0.0050, 0.0044 in sequence, middle panel: **P=0.0070, 0.0052, 0.0009 in sequence, right panel: **P=0.0035, 0.0014, 0.0079 in sequence; n = 3 independent experiments). b, c Western blot analysis of caspase-1 and IL-1β cleavage in mouse peritoneal macrophages from WT or Uaf1CKO mice after priming with LPS for the indicated time periods and then stimulated with ATP (b) or Nig (c) for 40 min. d ELISA analysis of serum levels of IL-1β, IL-6 and TNF from WT or Uaf1CKO mice after i.p. injection of LPS for 90 min (mean ± SEM, two-tailed t-test Uaf1CKO vs. WT, left panel: **P=0.0005, middle panel: *P=0.0321, right panel: **P<0.0001; n = 6 independent experiments). US, unstimulated; SN, supernatants; CL, cell lysates. Similar results were obtained from three independent experiments.

Fig. 2 UAF1 enhances NLRP3 expression. a Western blot analysis of NLRP3 (a), AIM2 and NLRC4 (c) in WT or Uaf1-deficient mouse peritoneal macrophages stimulated with LPS for the indicated time periods. b RT-PCR analysis of Nlrp3 mRNA expression in WT or Uaf1-deficient mouse peritoneal macrophages stimulated with LPS for 2 h (mean ± SEM, two-tailed t-test Uaf1CKO vs. WT, **P<0.0001; n = 3 independent experiments). d, e Western blot analysis of p-p65 in WT or Uaf1-deficient mouse peritoneal macrophages stimulated with LPS (d) or poly(I:C) (e) for the indicated time periods. f Analysis of NF-xB reporter activation using a Luciferase assay in HEK293T cells transiently transfected with NF-xB reporter plasmid together with MyD88 or TRIF, and UAF1 expression plasmid or empty control plasmid (mean ± SEM, two-tailed t-test UAF1 plasmid vs. empty control plasmid, **P=0.0024, <0.0001 in sequence; n = 6 independent experiments). Similar results were obtained from three independent experiments.

NLRP3 expression was ubiquitination-dependent, we examined the effects of USP1, USP12, and USP46 on NLRP3 expression and NLRP3 inflammasome activation. SiRNAs were used to suppress endogenous USP1, USP12, or USP46 expression. Usp1 siRNA 2, Usp12 siRNA 3, and Usp46 siRNA 1, which have higher efficiencies to inhibit the expression of respective targets (Supplementary Fig. 4a–c), were used in the following experiments. Usp1, Usp12, or Usp46 knockdown considerably inhibited IL-1β...
secretion, caspase-1 and IL-1β cleavage in LPS-primed and ATP-stimulated macrophages (Fig. 3a-d), indicating that USP1, USP12, and USP46 promoted NLRP3 inflammasome activation.

Although Usp1 knockdown had no effect on NLRP3 expression in unstimulated macrophages, it markedly attenuated LPS-induced NLRP3 expression (Fig. 3e). Furthermore, ML323, an allosteric and specific inhibitor of the UAF1/USP1 deubiquitinase complex, inhibited LPS-induced NLRP3 expression in a dose-dependent manner (Fig. 3f). However, both ML323 and Usp1 knockdown did not affect the expression of AIM2 and NLRC4 (Supplementary Fig. 4d, e). Interestingly, Usp12 and Usp46 knockdown markedly inhibited NLRP3 expression in both unstimulated and LPS-stimulated macrophages (Fig. 3g). In addition, both Usp12 and Usp46 knockdown significantly inhibited Nlrp3 mRNA expression; however, Usp1 knockdown had no effect on Nlrp3 mRNA expression (Fig. 3h). Collectively, these data indicate that USP1, USP12, and USP46 suppressed NLRP3 expression via different mechanisms.

**UAF1/USP12 and UAF1/USP46 complexes promote NLRP3 transcription.** Both Usp12 and Usp46 knockdown suppressed LPS-induced Il1b, Tnf, Il6, and Nlrp3 mRNA expression (Figs. 3h and 4a), suggesting that USP12 and USP46 may regulate the priming process of NLRP3 inflammasome activation. The transcription factor NF-κB is critical for NLRP3 expression. We thus examined the effects of USP12 and USP46 on NF-κB activation. Both USP12 and USP46 significantly enhanced MyD88-, TRAF6-, TAK1-, and p65-induced NF-κB luciferase reporter activation (Fig. 4b and Supplementary Fig. 5a), indicating that USP12 and USP46 promoted NF-κB activation by targeting p65 or downstream of p65. Moreover, both Usp12 and Usp46 knockdown considerably attenuated LPS-induced phosphorylation of p65 and total p65 expression (Fig. 4c, d), suggesting that p65 may be the target of USP12 and USP46. As parallel controls, USP1 overexpression had no effect on MyD88, TRAF6, TAK1, and p65-induced NF-κB luciferase reporter activation (Fig. 4b and Supplementary Fig. 5a). In addition, no difference in p65 expression was observed in Usp1 siRNA transfected macrophages (Supplementary Fig. 5b). Furthermore, Uaf1 deficiency also markedly inhibited p65 expression and phosphorylation of p65 (Figs. 2d, 4e). Collectively, these results indicate that UAF1/USP12 and UAF1/USP46 complexes promote NF-κB activation by enhancing p65 expression.

NF-κB subunit p65 can be inactivated and degraded by proteasomes. We then examined whether USP12/USP46 could target p65 to remove its polyubiquitin chains. P65 interacted with USP12, USP46 and UAF1 in both resting and LPS-stimulated macrophages, but exhibited no association with USP1 (Fig. 4f). Consistently, the interaction between exogenously expressed p65 and USP12/USP46/UAF1 was observed in HEK293T cells.
followed by IP with anti-p65, and subsequent probing with anti-Ub. Similar results were obtained from three independent experiments.

transiently transfected with Myc-p65, HA-Ub, or HA-tagged K48-linked ubiquitin (HA-K48-Ub), Flag-USP12, USP46, and Flag-UAF1 were subjected to IP.

ubiquitin mutant vectors K48 and K63, in which all their lysine residues were substituted with arginine except at positions 48 and 63, respectively, were used in the transfection assays. USP12, and USP46 markedly inhibited K48-linked ubiquitination of p65 (Fig. 4j), but had no effect on K63-linked ubiquitination of p65 (Supplementary Fig. 5d). Taken together, these results indicate that the UAF1/USP12 and UAF1/USP46 complexes remove K48-linked ubiquitination of p65 and enhance its expression, thereby promoting NLRP3 transcription.

(Fig. 4g). We then examined the effects of USP12/USP46/UAF1 on p65 polyubiquitination and observed that all the three could remove its polyubiquitination (Fig. 4h). Under physiological conditions, endogenous p65 was ubiquitinated upon LPS stimulation in macrophages, and Uaf1 deficiency markedly increased endogenous p65 ubiquitination (Fig. 4i). To study the forms of USP12/USP46/UAF1-mediated deubiquitination of p65, ubiquitin mutant vectors K48 and K63, in which all their lysine residues were substituted with arginine except at positions 48 and 63, respectively, were used in the transfection assays. USP12, and USP46 markedly inhibited K48-linked ubiquitination of p65 (Fig. 4j), but had no effect on K63-linked ubiquitination of p65 (Supplementary Fig. 5d). Taken together, these results indicate that the UAF1/USP12 and UAF1/USP46 complexes remove K48-linked ubiquitination of p65 and enhance its expression, thereby promoting NLRP3 transcription.
UAF1/USP1 interacts with NLRP3. USP1 enhanced NLRP3 protein expression, with no effect on Nlrp3 mRNA expression, suggesting that the UAF1/USP1 complex regulated posttranslational modification of NLRP3. We first examined the association between NLRP3 and UAF1/USP1. Confocal analysis demonstrated the colocalization between USP1/UAF1 and NLRP3 upon LPS stimulation (Fig. 5a, b). We expressed NLRP3 along with USP1 and UAF1 in HEK293T cells. NLRP3 could be immunoprecipitated with USP1 and UAF1 (Fig. 5c, d). In vitro binding assays demonstrated that NLRP3 could directly interact with USP1 and UAF1 (Fig. 5e, f). Next, to confirm the interaction between NLRP3 and UAF1/USP1 under physiological conditions, we assessed resting and LPS-stimulated macrophages by immunoprecipitation. An association between NLRP3 and USP1/UAF1 was detected in LPS-stimulated mouse peritoneal macrophages (Fig. 5g, h). Taken together, these data suggest that NLRP3 could directly interact with UAF1/USP1.

To search for the domains of NLRP3 responsible for the interaction with USP1 and UAF1, a series of Myc-tagged NLRP3 truncated mutants were constructed (Fig. 5i). USP1 and UAF1 were coimmunoprecipitated with NLRP3 wild-type (WT), LRR domain deletion mutant (ΔLRR), NACHT domain deletion mutant (ΔNACHT), PYD domain deletion mutant (ΔPYD), and NACHT and PYD domain deletion mutant (ΔNACHT ΔPYD).
Western blot analysis with anti-Ub. Cell lysates from WT or ML323 for 4 h and then stimulated with LPS for 4 h, followed by treatment with cycloheximide (CHX) for the indicated time periods. NLRP3 expression was immunoblotted with the indicated antibodies. The immunoprecipitates were denatured and reimmunoprecipitated with Myc antibody (two-step immunoprecipitation, Re-IP), and then immunoblotted with the indicated antibodies. Western blot analysis of extracts obtained from HEK293T cells transiently cotransfected with NLRP3-Myc, HA-UB, and Flag-USP1 (c) or Flag-UAF1 (d) were immunoprecipitated using Myc antibody. The immunoprecipitates were denatured and reimmunoprecipitated with Myc antibody (two-step immunoprecipitation, Re-IP), and then immunoblotted with the indicated antibodies. Western blot analysis of extracts from HEK293T cells transfected with HA-K48-Ub or HA-K63-Ub, Myc-NLRP3 and Flag-USP1 (e) or Flag-UAF1 (f), followed by IP with anti-Myc and subsequently probed with anti-HA. MEFs were pretreated with DMSO or ML323 for 4 h and then stimulated with LPS for 6 h. Cell lysates were immunoprecipitated with anti-NLRP3, followed by western blot analysis with anti-Ub. Cell lysates from WT or Uaf1 deficient mouse peritoneal macrophages stimulated with LPS were immunoprecipitated with anti-NLRP3, followed by western blot analysis with anti-Ub. Western blot analysis of extracts obtained from HEK293T cells transfected with HA-K48-Ub or HA-K63-Ub, Myc-NLRP3 and Flag-USP1 (e) or Flag-UAF1 (f), followed by IP with anti-Myc and subsequently probed with anti-HA. MEFS were pretreated with DMSO or ML323 for 4 h and then stimulated with LPS for 4 h, followed by treatment with cycloheximide (CHX) for the indicated time periods. NLRP3 expression level was quantitated by measuring band intensities using ‘Image’ software (Q). The values were normalized to actin (mean ± SEM, two-tailed t-test ML323 vs. DMSO, **P < 0.0001, respectively, n = 3 independent experiments). Western blot analysis of NLRP3 expression in WT and Uaf1 deficient mouse peritoneal macrophages pretreated with LPS for 8 h, followed by treatment with MG132 for 4 h before harvesting the cells. Similar results were obtained from three independent experiments.

(ANACHT), PYD domain deletion mutant (ΔPYD), LRR domain mutant (LRR) and NACHT domain mutant (NACHT) (Fig. 5), and Supplementary Fig. 6). However, the PYD domain mutant (PYD) lost the ability to bind USP1 and UAF1 (Fig. 5, k). These results indicate that the LRR and NACHT domains of NLRP3 interact with UAF1/USP1.

UAF1/USP1 removes NLRP3 ubiquitination and stabilizes NLRP3. NLRP3 could be ubiquitinated with both K48 and K63 linkage. We identified the deubiquitase complex UAF1/USP1 as NLRP3-associated proteins (Fig. 5), which prompted us to investigate whether the complex could remove the ubiquitination of NLRP3. Both USP1 and UAF1 considerably inhibited ubiquitination of NLRP3 (Fig. 6a, b). However, the USP1 point mutation (C90S) with substitution of the cysteine residue with serine at position 90, lost the ability to inhibit the polyubiquitination of NLRP3 (Fig. 6a), indicating that USP1 could remove the ubiquitination of NLRP3 through its deubiquitinating activity. Given that NLRP3 possibly forms a complex with other proteins, we performed a two-step immunoprecipitation assay (Re-IP) to exclude the effects of other proteins in the complex. In HEK293T cells, Myc-tagged NLRP3 was cotransfected with ubiquitin and USP1 or UAF1, respectively. The cell lysates were subjected to immunoprecipitation with anti-Myc, and then the immunoprecipitates were denatured, followed by reimmunoprecipitation with Myc antibody. Notably, NLRP3 polyubiquitination was markedly attenuated in the presence of USP1 or UAF1 (Fig. 6c, d). Furthermore, USP1 inhibited NLRP3

**Fig. 6** UAF1/USP1 removes NLRP3 ubiquitination and stabilizes NLRP3. a, b Western blot analysis of extracts obtained from HEK293T cells transfected with HA-Ub, Myc-NLRP3, Flag-USP1 WT, and USP1 C90S (a) or Flag-UAF1 (b), followed by IP with anti-Myc, and then probed with anti-HA. c, d Extracts obtained from HEK293T cells transiently cotransfected with NLRP3-Myc, HA-UB, and Flag-USP1 (c) or Flag-UAF1 (d) were immunoprecipitated using Myc antibody. The immunoprecipitates were denatured and reimmunoprecipitated with Myc antibody (two-step immunoprecipitation, Re-IP), and then immunoblotted with the indicated antibodies. e, f Western blot analysis of extracts from HEK293T cells transfected with HA-K48-Ub or HA-K63-Ub, Myc-NLRP3 and Flag-USP1 (e) or Flag-UAF1 (f), followed by IP with anti-Myc and subsequently probed with anti-HA. g MEFs were pretreated with DMSO or ML323 for 4 h and then stimulated with LPS for 6 h. Cell lysates were immunoprecipitated with anti-NLRP3, followed by western blot analysis with anti-Ub. h Cell lysates from WT or Uaf1 deficient mouse peritoneal macrophages stimulated with LPS were immunoprecipitated with anti-NLRP3, followed by western blot analysis with anti-Ub. i Western blot analysis of extracts obtained from HEK293T cells transfected with HA-K48-Ub or HA-K63-Ub, Myc-NLRP3 and Flag-USP1 (e) or Flag-UAF1 (f), followed by IP with anti-Myc and subsequently probed with anti-HA. j Western blot analysis of NLRP3 and caspase-1 expression in mouse peritoneal macrophages pretreated with DMSO or ML323 for 4 h and then stimulated with LPS for 4 h, followed by treatment with cycloheximide (CHX) for the indicated time periods. NLRP3 expression level was quantitated by measuring band intensities using ‘Image’ software (Q). The values were normalized to actin (mean ± SEM, two-tailed t-test ML323 vs. DMSO, **P < 0.0001, respectively, n = 3 independent experiments). k Western blot analysis of NLRP3 expression in WT and Uaf1 deficient mouse peritoneal macrophages pretreated with LPS for 8 h, followed by treatment with MG132 for 4 h before harvesting the cells. Similar results were obtained from three independent experiments.
polyubiquitination with K48-linkage, but not with K63-linkage (Fig. 6c), indicating that USP1 selectively removed K48-linked polyubiquitination of NLRP3. Consistently, UAF1 also inhibited K48-linked polyubiquitination of NLRP3 (Fig. 6f). Under physiological conditions, endogenous NLRP3 was ubiquitinated upon LPS stimulation. Moreover, polyubiquitination of NLRP3 was enhanced in ML323-treated mouse embryonic fibroblasts (MEFs) (Fig. 6g) and Uaf1-deficient macrophages (Fig. 6h). Collectively, these data indicate that UAF1/USP1 directly eliminates K48-linked polyubiquitination of NLRP3 through its deubiquitinase activity.

K48-linked protein ubiquitination leads to degradation of the corresponding proteins. Both UAF1 and USP1 enhanced LPS-induced NLRP3 expression. We then investigated the effects of UAF1/USP1 complex on NLRP3 protein degradation by cycloheximide (CHX) chase experiment. ML323 treatment significantly promoted NLRP3 protein degradation, with no effect on caspase-1 (Fig. 6i, j). In addition, Uaf1 deficiency resulted in a loss of the inhibitory effects on NLRP3 expression in MG132-treated macrophages (Fig. 6k). Taken together, these data indicate that the USP1/UAF1 deubiquitinase complex targets NLRP3 and inhibits its degradation, thus facilitating NLRP3 inflammasome activation.

ML323 and Uaf1 deficiency ameliorate NLRP3-dependent inflammation. Next, we investigated whether ML323, a selective UAF1/USP1 inhibitor, could suppress NLRP3 inflammasome activation. ML323 treatment attenuated NLRP3-dependent IL-1β secretion and caspase-1 cleavage in macrophages (Fig. 7a, b). ML323 is known to be active in vivo36. We then examined the physiological relevance of the UAF1/USP1 complex in inflammation in vivo using ML323. ML323-treated mice produced significantly less IL-1β and TNF in sera after i.p. injection of LPS than control mice, while no difference in IL-6 secretion was observed (Fig. 7c).

Folic acid (FA)-induced acute tubular necrosis (ATN) is tightly associated with NLRP3 inflammasome activation37. To evaluate the roles of ML323 in FA-induced ATN, mice were i.p. injected with ML323 followed by an FA injection. ML323 treatment considerably reduced IL-1β secretion in sera (Fig. 7d) and NLRP3 expression in the kidneys (Fig. 7e). Renal inflammation and edema were greatly ameliorated in the kidneys of ML323-treated mice (Fig. 7f, g). Concordantly, ML323-treated mice were found to be more resistant in survival assays upon FA injection (Fig. 7h).

Therefore, as a selective UAF1/USP1 complex inhibitor, ML323 is known to be active in vivo36. We then examined the secretion and caspase-1 cleavage in macrophages (Fig. 7a, b). ML323-treatted mice were found to be more resistant in survival assays upon FA injection (Fig.7h). On the other hand, UAF1/USP1 complex selectively interacts with NLRP3, removes NLRP3 K48-linked ubiquitination and prevents NLRP3 from proteasomal degradation. Therefore, UAF1 facilitates NLRP3 inflammasome activation by enhancing IL-1β and NLRP3 expression at the mRNA and protein levels. Previously, we reported that LPS induced UAF1 expression in macrophages36. Thus, UAF1 might be a feedback enhancer of NLRP3 inflammasome activation.

NLRP3 protein expression is considered to be a rate-limiting step and causes subsequent NLRP3 inflammasome activation. In resting macrophages, NLRP3 expression is relatively low; hence, NLRP3 inflammasome assembly is hardly induced. However, following stimulation with exogenous and endogenous factors, including TLR agonists and proinflammatory cytokines, NLRP3 expression is dramatically induced to establish quick responses to NLRP3 activators. At the transcriptional level, NF-κB is critical for Nlrp3 mRNA expression34. Zn finger protein A20 deubiquitinates NF-κB, thereby signaling molecules to suppress NF-κB activation and NLRP3 transcription39. Aryl hydrocarbon receptor (AhR) binds to the xenobiotic response elements (XREs) located within two NF-κB binding sites in the NLRP3 promoter and attenuates NLRP3 transcription and NLRP3 inflammasome activation40. In this study, we demonstrate that UAF1/USP12 and UAF1/USP46 complexes target p65 and promote NF-κB activation, resulting in the enhancement of NLRP3 transcription and NLRP3 inflammasome activation. In addition, the UAF1/USP12 and UAF1/USP46 complexes also enhance the transcription of proinflammatory cytokines, including pro-IL-1β, TNF, and IL-6, which further promote NLRP3 inflammasome activation. At post-transcriptional level, multiple E3 ubiquitin ligases promote NLRP3 ubiquitination and protein degradation, which limit NLRP3 inflammasome activation. In this study, we observed that the UAF1/USP1 deubiquitinase complex inhibits proteasomal degradation of NLRP3 and enhances NLRP3 inflammasome activation. Deubiquitination of NLRP3 is required for optimal NLRP3 inflammasome activation. Our results indicate that NLRP3 deubiquitination is important for the maintenance of NLRP3 protein level, thus providing a mechanism to elucidate the indispensable roles of ubiquitination in NLRP3 inflammasome activation.

UAF1, as a co-factor of USP1, USP12, and USP46, could enhance their deubiquitinase activity by forming stable UAF1/USP1 protein complexes41. UAF1/USP1, UAF1/USP12, and UAF1/USP46 complexes play vital roles in DNA repair processes and tumor pathogenesis. Here, we demonstrated that UAF1 works together with USP12/USP46 and USP1 to facilitate NLRP3 and pro-IL-1β expression, and license NLRP3 inflammasome activation. As NLRP3 inflammasome needs two signals (priming signal and activation signal) to be activated, it acts just like a precise lock. Under certain circumstances, UAF1 and its partners (USP1/USP12/USP46) work as multiple keys to unlock and tightly control the activation of NLRP3 inflammasome at different levels (Supplementary Fig. 7).

Inappropriate NLRP3 inflammasome activation has been implicated in a variety of diseases, which makes NLRP3 an attractive drug target. Many inhibitors of the NLRP3 pathway have been developed and extensively investigated in multiple NLRP3 inflammasome-related disease models. For example, MCC950, a best studied NLRP3 inhibitor, displays efficacy in a
wide range of NLRP3-dependent murine disease models. CY-09, another selective NLRP3 inhibitor, is also found to be efficacious in a mouse model of cryopyrin-associated periodic syndromes (CAPS). In this study, we identified ML323 as a novel NLRP3 inflammasome inhibitor. ML323 is a specific inhibitor of the UAF1/USP1 complex, with no inhibitory effects on both UAF1/USP12 and UAF1/USP46 complexes. ML323 inhibits NLRP3 protein expression, suppresses NLRP3 inflammasome activation, thereby ameliorating NLRP3-dependent inflammation both in vitro and in vivo. Our results suggest that ML323 might be a promising candidate for the treatment of inflammatory diseases caused by aberrant NLRP3 activity.

In conclusion, by identifying UAF1 as a critical enhancer of NLRP3 and pro-IL-1β expression, this study provides insights into the mechanism of NLRP3 inflammasome activation. UAF1 enhances NLRP3 expression at both the mRNA and protein levels, as well as pro-IL-1β transcription through recruiting USP11/12/46. The UAF1/USP1 complex directly targets NLRP3 and inhibits its ubiquitination degradation. The UAF1/USP12/USF1 and UAF1/USP46 complexes suppress the ubiquitination degradation of p65, promote NF-kB activation, and enhance NLRP3 and pro-IL-1β transcription. These results indicate that the UAF1 deubiquitination complexes enhance NLRP3 and pro-IL-1β expressions via targeting NLRP3 and p65, and thus facilitates NLRP3 inflammasome activation.

**Methods**

**Mice.** Uaf1fl/fl mice on a C57BL/6 background were generated by Cyagen Biosciences Inc. (Guangzhou, China) using CRISPR-Pro technology. Lys2-Cre mice were from Jackson Laboratory. Uaf1fl/fl/Lys2-Cre mice were crossed with Lyz2-Cre transgenic mice to obtain Uaf1fl/fl/Lys2-Cre mice with Uaf1 deficiency in myeloid cells, resulting in Uaf1-deficient mice (Uaf1CKO) mice with 984 missing base pairs (6350-7334, containing the third exon of Uaf1 gene NM_026236.3), which caused a frameshift. For animal experiments with Uaf1fl/fl mice, littermate controls with normal Uaf1 expression (Uaf1+/+) were used. C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals; approval was obtained from the Scientific Investigation Board of Medical School of Shandong University (Jinan, Shandong Province, China). All the animals were generated in specific pathogen-free (SPF) levels with 40 °C and 12 h of dark at 21 °C.

**Reagents.** ATP, MGI322, LPS (Escherichia coli, 055:B5), poly(I:C), folic acid, anti-HA (HA-7, cat. H3663, 1:1000 for WB), anti-Myc (9E10, cat. M4439, 1:1000 for WB), and anti-Flag (M2, cat. F1804, 1:1000 for WB, 1:400 for IP) were purchased from Sigma-Aldrich. Anti-Myc (9E10, cat. TA180121Z, 1:400 for IP) and anti-HA (C9051, cat. TA180128-1, 1:400 for IP) were from OriGene. Poly(DA) andFLAG were purchased from InvivoGen. ML323 was obtained from Selleck Chemicals. GHX was purchased from APEXIBio Technology. Anti-NLRP3 (polyclonal, mouse) was purchased from Abcam. 

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whole-cell extracts were lysed in IP buffer containing 1.0% (v/v) Nonidet P 40, 50 mM Tris pH 7.2, 150 mM NaCl, and a protease inhibitor ‘cocktail’ (Merck, cat. p8340). After centrifugation for 10 min at 14,000 g, supernatants were collected and incubated with protein G Plus-Agarose.
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Author contributions

W.Z. designed and supervised the research; H.S., C.Z., Z.Y., Q.L., R.Y., Y.Q., and M.J. performed the experiments; H.S., C.Z., and W.Z. analyzed the data and wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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