TRAF3 promotes ROS production and pyroptosis by targeting ULK1 ubiquitination in macrophages

Yang Shen | Wen-wen Liu | Xiu Zhang | Jian-guo Shi | Shan Jiang | Lishuang Zheng | Yan Qin | Bin Liu | Jian-hong Shi

Abstract
Disrupted mitochondrial function and reactive oxygen species (ROS) generation cause cellular damage and oxidative stress-induced macrophage inflammatory cell death. It remains unclear how mitochondrial dysfunction relates to inflammasome activation and pyroptotic cell death. In this study, we demonstrated that tumor necrosis factor receptor-associated factor 3 (TRAF3) regulates mitochondrial ROS production and promotes TLR agonist LPS plus nigericin (LPS/Ng)-induced inflammasome and pyroptosis in mouse primary macrophages and human monocyte THP-1 cells. Co-IP assays confirmed that TRAF3 forms a complex with TRAF2 and cIAP1 and mediates ubiquitin and degradation of Unc-51 like autophagy activating kinase 1 (ULK1). Moreover, knockdown of ULK1 in THP-1 cells significantly promoted LPS/Ng-induced inflammasome by activating caspase 1 and mature IL-1β. Apoptosis inducing factor (AIF) translocation from mitochondrial to nuclear was observed in ULK1-deficient THP-1 cells under LPS/Ng stimulation, which mediates LPS/Ng-induced cell death in ULK1 deficient macrophages. In conclusion, this study identified a novel role of TRAF3 in regulation of ULK1 ubiquitination and inflammasome signaling and provided molecular mechanisms by which ubiquitination of ULK1 controls mitochondrial ROS production, inflammasome activity, and AIF-dependent pyroptosis.

KEYWORDS
cell death, inflammasome, macrophages, mitochondrial, reactive oxygen species
In response to pathogen infection, inflammatory reactions are activated through recognizing pathogens by pattern recognition receptors (PRRs) in macrophages, such as toll-like receptors (TLRs). Disordered inflammatory response is known to be one of the most important causes of autoimmune diseases, such as inflammatory bowel diseases, systemic sclerosis, polymyositis, and diabetes. Thus, defining the mechanisms of inflammation regulation is then considered of great importance.

Macrophages play various roles in different stages of diseases due to their heterogeneity. At the early stage of pathogens, activated macrophages release a lot of pro-inflammatory cytokines, including interleukin (IL)-1β and IL-18. Macrophages undergo inflammatory programmed cell death, called pyroptosis, at the late stage of inflammatory response under certain pathological stimulations. Macrophages primed by TLR agonists followed by nigericin or ATP stimulation induces the formation of inflammasome and accelerate pyroptotic cell death. The pyroptotic cells are characterized as mature caspase 1 production and propidium iodide (PI)-positive staining. Moreover, releasing of a large amount of certain proteins, such as IL-1β and IL-18, from cytosol to extracellular are also considerable evidence of pyroptotic cells. Mitochondrial dysfunction and reactive oxygen species (ROS) production are also associated with macrophage inflammasome activation, which may directly cause cellular damage, oxidative stress, and pyroptotic process. Although accumulating evidence demonstrate significant role of inflammasome signaling and pyroptosis in inflammatory diseases, it remains unclear how mitochondrial dysfunction relates to inflammasome activation and pyroptotic cell death.

Tumor necrosis factor receptor-associated factor 3 (TRAF3), a TRAF family member with E3 ligase activity, functions as an important mediator of innate immune receptor signaling. TRAF3 downregulates noncanonical NF-κB signaling by mediating TRAF3-TRAF2-cIAP complex-dependent NF-κB inducing kinase (NIK) K48-linked ubiquitination and degradation. TRAF3 controls c-Rel and IRF5 degradation via TRAF3-TRAF2-cIAP complex and negative regulates IRF5/c-Rel signaling events involving pro-inflammatory factor production in TLR stimulated macrophages. Several recent reports indicated that TRAF3 participates ASC K63-linked ubiquitination and promotes virus-induced inflammasome activation. In the present study, we provided evidence that TRAF3 is involved in mitochondrial ROS production and regulates TLR4 agonist LPS plus nigericin-induced inflammation and pyroptotic cell death. Deficiency in TRAF3 promotes unc-51 like autophagy activating kinase 1 (ULK1) accumulation and reduces inflammasome response and apoptosis inducing factor (AIF)-dependent cell death, which define a new signaling cascade for TLR stimulated inflammasome activation.

2 | METHODS

2.1 | Mice

Traf3-flox mice (C57BL/6 background) were provided by Dr Robert Brink (Garvan Institute of Medical Research). These mice were crossed with lysozyme 2-Cre (Lyz2-Cre) mice (C57BL/6 background, Jackson Laboratories) to produce myeloid cell-conditional knockout mice (TRAF3 Traf3fl/fl-lyz2Cre/+, termed Traf3MKO) and age-matched wild-type mice (Traf3fl/fl-lyz2+/, termed WT). The mice were maintained in specific pathogen-free facility of Hebei University, and all animal experiments were conducted in accordance with guidelines and regulations approved by the Institutional Animal Care and Use Committee of Hebei University. All animal experiments were approved by the Animal Research Ethics Committee of the authors’ institution.

2.2 | Antibodies and reagents

Antibodies for TRAF2 (C-20, 1:1,000), TRAF3 (H-122, 1:1,000), ubiquitin (P4D1, 1:1,000), c-Myc (9E10, 1:1000), caspase 1 p10 (1:1,000), caspase 1 p20 (1:1,000), caspase 2 (12586, 1:1,000), caspase 7 (9492, 1:1000), PARP (9532, 1:1,000), Caspase 9 (9508, 1:1,000), Caspase 3 (9665, 1:1,000), Caspase 8 (9746, 1:1,000) were purchased from Cell Signaling Technology. Antibodies for mouse caspase 1 p10 (1:1,000) and human caspase 1 p20 (1:1,000) were purchased from AdipoGen. Antibodies for mouse IL-1β (AF-401-NA, 1:1,000) and human IL-1β (AF-201-NA, 1:1,000) were purchased from R&D. Antibodies for GSDMD (ab210070, 1:1,000), cIAP1 (ab108361, 1:5,000), and anti-ubiquitin (linkage-specific K48) antibody (ab140601) were purchased from Abcam. Anti-Actin (A2228, 1:10,000), Horseradish peroxidase-conjugated anti-Flag antibody (M2) and anti-LC3B (L7543, 1:5,000) was from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-HA antibody (3F10) was purchased from Roche. Horseradish peroxidase-conjugated anti-V5 antibody were purchased from Thermo Fisher Scientific. The fluorochrome-labeled antibodies specific to mouse proteins APC-conjugated anti-CD11b and PE-conjugated anti-F4/80 and their corresponding isotype controls were purchased from eBioscience (San Diego, CA, USA). MitoTracker Deep Red FM (MitoTracker M22426), MitoSox Red Mitochondrial Superoxide Indicator (MitoSox, M36008), SYTOX Red Dead Cell Stain were purchased from MitoSox Red Mitochondrial Superoxide Indicator (MitoSox, M36008).
Thermo Fisher Scientific. LPS (derived from Escherichia coli strain 0127: SB8), nigericin, Ac-YVAD-CMK, Z-Leu-Leu-Leu-al (MG132), N-acetyl-l-cysteine (NAC), and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich. Flagellin was from AdipoGen (San Diego, CA, USA). Poly(deoxyadenylic-thymidylic) acid [poly(dA:dT)] was purchased from InvivoGen (San Diego, CA, USA).

2.3 Plasmids and shRNAs

Expression vectors encoding human TRAF2 and TRAF3 and truncation mutants of TRAF3 (88-C, 333-C, and 1-448), myc-ULK1, Flag-clAP1, Myc-clAP1 H588A, and HA-Ub were purchased from Addgene. pGIPZ lentiviral vectors encoding shRNAs specific targeting human ULK1 or a non-targeting control shRNA (shNT) were purchased from GE healthcare (CO, USA). Lentiviral vectors silencing human TRAF3 were purchased from Sigma-Aldrich. LV3 lentiviral vector silencing human TRAF2 and LV10 lentiviral vector silencing human AIF were purchased from GenePharma (Suzhou, China). The sequences of shRNAs listed in Table 1. All DNA constructs used were verified by DNA sequencing.

2.4 Gene silencing and overexpression

For gene silencing, lentiviral particles were prepared by transfecting HEK293 cells with lentiviral vectors encoding gene specific targeting shRNAs or control shRNA along with packaging plasmids. The packaged viruses were then used to infect the indicated cells, followed by selection of the infected cells by puromycin selection or flow cytometric cell sorting based on vector carried GFP or RFP expression.

2.5 Cell culture, Macrophage preparation, and inflammasome activation

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% of fetal bovine serum (FBS), 100 Units of penicillin and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% of CO₂. Human monocyte THP-1 cells were maintained in RPMI 1640 medium containing 10% of FBS and penicillin-streptomycin. At the end of the incubation period, the cells were induced differentiation to macrophages overnight in RPMI 1640 medium containing 0.5% of FBS and PMA (30 ng/mL) before stimulation.

Bone marrow derived macrophages (BMDMs) were generated by cultivating bone marrow cells isolated from the femurs of indicated young adult WT or TRAF3MKO mice in growth medium supplemented with M-CSF conditional medium for 5 days. For peritoneal macrophages (PEMs) isolation, WT or TRAF3MKO mice were injected ip with 3 mL of thioglycolate medium (3% of solution, 211716, Sigma). Mice were killed 3 days later and the peritoneal cavity was lavaged with 8 mL of RPMI 1640 medium. The medium recovered from mice and cells were collected by centrifugation and plated onto 6-well plates in a density of 1 x 10⁶ cells/mL. Macrophages were allowed to adhere for 2 hours (37°C, 5% of CO₂), and then, washed with fresh medium to remove unattached cells and incubated overnight. BMDMs and PEMs were starved overnight in medium supplemented with 0.5% of FBS before being stimulated.

To stimulate inflammasome activation, medium was replaced with fresh medium and macrophages were primed with LPS (1 μg/mL) for 3 hours followed by nigericin (Ng, 5 μg/mL), ATP (5 mM), poly(dA:dT) (2 μg/mL), or flagellin (3 μg/mL) simulation for an additional time as indicated. Poly(dA:dT) was transfected with Exfect Transfection Reagent (Vazyme, Nanjing, China).

2.6 Flow cytometry

The cells were stained with indicated fluorescence labelled antibodies and subjected to flow cytometry using FACS Calibur (BD Bioscience). For PEMs, peritoneal lavage fluid from WT or TRAF3MKO mice were stained with APC-conjugated anti-CD11b and PE-conjugated anti-F4/80. Mitochondrial mass was measured by fluorescence levels
upon staining with MitoTracker at 50 nM for 15 minutes at 37°C. Mitochondria-associated ROS levels were measured by incubating cells with MitoSox at 5 μM for 15 minutes at 37°C. Cells were then washed with PBS solution and re-suspended in cold PBS solution for Flow cytometry analysis.

2.7 | Real-time quantitative RT-PCR

Total RNA was isolated from macrophages using TRIzol reagent (Invitrogen) and subjected to cDNA synthesis using HiScript III RT SuperMix for qPCR Kit (Vazyme Biotech, Nanjing, China). Real-time quantitative PCR (qRT-PCR) was performed using and ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China). The expression of individual genes was calculated by a standard curve method and was normalized to the expression of β-Actin. The primers used: mULK1 forward: AAGTTCGAGTTCTCTCGCAAG; mULK1 reverse: CGATGTTTTCGTGCTTTAGTTCC.

2.8 | Immunoblot (IB), co-immunoprecipitation (coIP) Assay, and ELISA

Whole-cell lysates or subcellular extracts were prepared and subjected to IB and coIP assays. In brief, samples were separated by dodecyl sulfate sodium-polyacrylamide gel electrophoresis, transferred to PVDF membranes and blocked with 5% of nonfat milk. The membranes were then washed and incubated with a specific primary antibody. Horseradish peroxidase-conjugated secondary antibody was applied and immunoreactive bands were visualized by ECL chemiluminescent detection and exposure to X-ray film. For coIP assays, the cells were lysed in a buffer containing 50 mM of Tris-HCl, pH7.4, 150 mM of NaCl, 1% of NP-40, 0.5% of Na-deoxycholate, 1 mM of EDTA, and a protease inhibitor mixture. Cell lysates were first pre-cleared with protein A-agarose (50% v/v, Santa Cruz). The supernatants were immunoprecipitated with indicated antibody for 1 hour at 4°C, followed by incubation with protein A-agarose overnight at 4°C. Then, the protein A-agarose-antigen-antibody complex pellet were washed five times and were analyzed by IB. Mouse IL-1β ELISA Kit (Abclonal, Wuhan, China) was used to quantify production of IL-1β in supernatant of BMDMs upon LPS/Ng stimulation and mouse peritoneal lavage fluid.

2.9 | Ubiquitination assays

Cells were pretreated with MG132 for 2 hours, and then, lysed with a NP-40 lysis buffer (50 mM of Tris-HCl, pH7.5, 120 mM of NaCl, 1% of NP-40, 1 mM of EDTA, 1 mM of DTT) containing 6 M urea and protease inhibitors. The indicated proteins were isolated by immunoprecipitation (IP) with anti-ULK1 antibody and the ubiquitinated ULK1 was detected by IB using anti-ubiquitin (P4D1, Santa Cruz Biotech, USA) and anti-ubiquitin (linkage-specific K48, ab140601, Abcam, USA) antibodies. For transfection models, expression vectors encoding myc-ULK1 was transfected into HEK293 cells along with HA-ubiquitin and other indicated vectors. The cells were pretreated with MG132 for 2 hours, and then, lysed for IP followed by detecting ubiquitination by IB using anti-HA antibody.

2.10 | Cell viability assessment and cell death assays

After cells were stimulated with LPS/Ng, cell viability of macrophages was measured by the MTT assay. Twenty microliters of the MTT solution (5 mg/mL; Sigma-Aldrich, USA) was added to each well for an additional 2 hours. After careful removal of the medium, 100 μL of DMSO was added to dissolve the dark blue formazan crystals. Finally, absorbance at 570 nm was measured using a Bio Tek Epoch Spectrophotometer (Bio Tek Instruments, Inc, Winooski, USA). For cell death assays, cells were incubated with SYTOX Red Dead Cell Stain (S34859, Thermo Fisher Scientific, USA) for 10 minutes, and then, subjected to flow cytometry to quantify the apoptotic cell population. Lactate dehydrogenase (LDH) release was assessed in cell-free medium following the manufacturer’s instructions of Cytotoxicity Detection (LDH) Kit (Roche Applied Science, Mannhein, Germany) to evaluate cell death.

2.11 | In Vivo monosodium urate (MSU)-induced peritonitis mouse model

To analyze NLRP3-induced inflammasome in vivo, MSU-induced peritonitis mouse model was performed. C57BL/6 mice were injected ip with 1 mg MSU in 200 μL of PBS and the control mice received PBS. The peritoneal lavage samples were obtained 5 hours after MSU injection by washing the peritoneal cavity with 2 mL of cold PBS with 1% of FBS. Peritoneal samples were centrifuged and total cells were counted. Subsequently, cells were stained with CD11b (Percp-cy5.5 rat anti-mouse CD11b, BD Pharmingen, 550993), F4/80 (Alexa Fluor 647 rat anti-mouse F4/80, BD Pharmingen, 565853) and Gr-1 (PE rat anti-mouse LY-6G and LY-6C, BD Pharmingen, 553128) antibodies and analyzed using flow cytometry (FACS Calibur (Becton Dickinson, San Diego, CA, USA). The number of neutrophils was calculated as total cells multiplied by CD11b+/Gr-1+/F4/80- cell ratio. The supernatant of peritoneal lavage fluid
was used to evaluate IL-1β production using Mouse IL-1β ELISA Kit (Abclonal, Wuhan, China).

2.12 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. Two-tailed paired Student’s t tests or one-way ANOVA analysis with Tukey’s multiple comparisons were used according to the number of groups compared. P values < 0.05 were considered significant.

3 | RESULTS

3.1 | TRAF3 deficiency reduces mitochondrial ROS and inhibits LPS/Ng-induced pyroptotic cell death in macrophages

To define the macrophage inflammatory function of TRAF3, we generated myeloid cell-conditional knockout mice (TRAF3\textsuperscript{MKO}) and age-matched WT mice. Immunoblotting assays showed TRAF3 was abolished in PEMs and BMDMs (Figure 1A). ROS are known to

![Graph showing statistical analysis results](image-url)
promote inflammation and inflammatory cell death. A main source of ROS is mitochondria, and abnormal ROS production is often associated with mitochondrial dysfunction and cell death.\(^{23}\) TRAF3 deficiency in PEMs significantly reduced mitochondrial ROS production measured with MitoSox (Figure 1B-D).

Disrupted mitochondrial ROS generation may cause cellular damage and oxidative stress-induced macrophage inflammatory cell death. Mouse PEMs and human monocyte THP-1 cells were then treated with LPS plus nigericin (LPS/Ng), classical inducers of inflammasome and pyroptotic cell death, and cell viability and pyroptotic cell death were then examined. LPS/Ng treatment reduced cell viability in WT PEMs, which was partially abolished in TRAF3\(^{3\text{MKO}}\) PEMs (Figure 1E). Furthermore, LPS/Ng-induced pyroptosis significantly decreased in TRAF3-knockdown human monocyte THP-1 cells (Figures 1F,G and S1). To further investigate the in vivo biological function in mediating inflammasome activation, an MSU-induced peritoneal inflammasome mouse model was performed and the production of IL-1β and the influx of neutrophils into the peritoneal cavity were determined. Compared with the WT mice, TRAF3 deficiency resulted in a significant decrease in IL-1β production (Figure 1H) and neutrophil infiltration (Figures I,J and S2). Collectively, these data suggested that TRAF3 regulates mitochondrial ROS production, NLRP3 inflammasome activation, and pyroptotic cell death.

### 3.2 ULK1 upregulates in TRAF3\(^{3\text{MKO}}\) macrophages and participates in pyroptotic cell death

ROS and autophagy are both key factors in breaking cellular homeostasis, resulting in oxidative stress, mitochondrial dysfunction and cell death.\(^{24,25}\) Autophagy plays a fundamental role in removing damaged mitochondria to prevent abnormal ROS production. To further analyze the molecular mechanisms of TRAF3 in LPS/Ng-induced pyroptotic cell death, we investigated the expression level of ULK1, a key regulator of autophagy, in LPS/Ng treated macrophages. Interestingly, TRAF3 protein level slightly decreased and ULK1 protein level increased significantly after LPS/Ng administered for 30 or 60 minutes in BMDMs (Figure 2A). Interestingly, compared with the WT cells, we also observed a higher ULK1 expression level in untreated TRAF3-deficient BMDMs, which dramatically increased after LPS/Ng stimulation (Figure 2A). Moreover, the basal expression levels of ULK1 in TRAF3\(^{3\text{MKO}}\) BMDM and PEM were distinctly higher than that in WT macrophages, respectively (Figure 2B), while there is no significant difference in ULK1 mRNA levels between WT and TRAF3\(^{3\text{MKO}}\) macrophages (Figure 2C). These data suggested a potential role of ULK1 in TRAF3-mediated ROS production and pyroptotic cell death.

To determine the role of ULK1 in LPS/Ng-induced ROS production and cell death, human monocyte THP-1 was used to generate ULK1 stable knockdown cell lines (shULK1#1 and shULK1#2), and nontargeting shRNA (shNT) transduced THP-1 cells was as a negative control. We found that the majority of shNT THP-1 cells had a low content of mitochondrial ROS, whereas the entire population of shULK1#1 and shULK1#2 THP-1 cells exhibited higher contents of mitochondrial ROS, suggesting abnormal ROS production (Figure 2D). Consistently, compared with control group, the ULK1-knockdown THP-1 cells also had profoundly increased mitochondrial content, indicative of mitochondrial dysfunction (Figure 2E). Following LPS/Ng stimulation in control and ULK1-knockdown THP-1 cells, ULK1 knockdown resulted in a sharply elevation in pyroptotic cell death (Figure 2F). Moreover, pretreated cells with N-Acetyl-L-cysteine (NAC), ROS scavenger, largely, although not completely, rescued LPS/Ng-induced cell death in ULK1 knockdown THP-1 cells (Figure 2G). These results suggest that ULK1 deficiency in THP-1 cells causes abnormal mitochondrial ROS production, which contributes to the LPS/Ng-induced cell death.

### 3.3 TRAF3 deficiency blockades ubiquitin-dependent ULK1 degradation

TRAF3 is known as an E3 ubiquitin ligase and participates in multiple biological processes.\(^{26}\) In searching for the molecular mechanism by which TRAF3 regulates ULK1 protein level, we found that TRAF3 interacts with ULK1 in THP-1 cells (Figure 3A). To confirm and map the domain of TRAF3-ULK1 physical interaction in mammalian cells, a panel of deletion mutants of TRAF3 was subjected to ULK1-binding assays using transfected HEK293 cells. WT and two TRAF3 mutants lacking the N-terminal portion (1-448) and C-terminal portion (88-C) were capable of ULK1 interaction (Figure 3C, left panel, lanes 2, 3, and 5). On the contrary, a TRAF3 mutant lacking the ZF domain (333-C) was unable to bind to ULK1 (lane 4), suggesting that ZF domain of TRAF3 is necessary for TRAF3/ULK1 association. TRAF3 deficiency greatly promoted the expression of ULK1 at protein level but not mRNA level (Figure 2B,C), suggesting a posttranslational regulation. To examine whether ULK1 degradation is mediated through its TRAF3-associated ubiquitination, we analyze the polyubiquitination of ULK1 in MG132 treated WT and TRAF3\(^{3\text{MKO}}\) macrophages. Indeed, in untreated cells, ubiquitinated forms of ULK1 was hardly detected (Figure 3D, lane 2 and 3). When the degradation of
ULK1 was inhibited by MG132, the steady level of ULK1 in total cell lysates was remarkably increased, which is almost similar to that of the TRAF3MKO cells (Figure 3D, lower panel, lane 2, 4, and 5, Figure S3). Furthermore, the ubiquitinated forms of ULK1 could be readily detected in MG132 treated cells (Figure 3D, lane 4), whereas the polyubiquitination of ULK1 was not significantly detected in TRAF3MKO macrophages (lane 5). These results suggested that TRAF3/ULK1 interaction mediates ULK1 degradation through the proteasome.

### 3.4 LPS/Ng-triggered inflammasome inhibits ubiquitin-dependent ULK1 degradation in macrophages

Inflammasomes are innate immune signaling platforms controlling inflammatory, which are classically activated by LPS plus nigericin or ATP. Although we have found that ULK1 undergoes rapid turnover in WT macrophages, it is still unknown whether ULK1 degradation can be blocked during inflammasome activation. After BMDMs...
were treated with LPS/ATP or LPS/Ng for 30 minutes, respectively. ULK1 protein level significantly increased (Figure 4A). Compared to the LPS-treated BMDMs, K48-linked polyubiquitinated ULK1 obviously decreased in LPS/ATP- and LPS/Ng-cells (Figure 4B), which suggested ULK1 undergoes inhibition of ubiquitin-dependent degradation during inflammasome activation in macrophages. We also examined TRAF3 levels, and in contrast of ULK1 upregulation, TRAF3 significantly decreased in LPS/Ng-stimulated cells (Figure 4C). TRAF3 often associates with E3 ubiquitin ligase TRAF2 and cIAP1 to form a complex that mediates target protein ubiquitination and degradation. We also find that the protein level of cIAP1 reduced, parallel with that of TRAF3 (Figure 4C), suggesting functional involvement of cIAP1 in inflammasome-associated ULK1 expression.
3.5 | cIAP1 is involved in ULK1 degradation

Although recent data suggested important role of TRAF3 and TRAF2 in mediating cIAP1 association and ubiquitination with target protein, such as NIK,\textsuperscript{27} it has been still unknown whether this E3 ubiquitin ligase complex also mediates additional substrate proteins. To further determine function of cIAP1 in ULK1 stabilization, we transfected HEK293 cells with an increasing amount of Flag-cIAP1. As expected, the expression of ULK1 in protein level significantly decreased, inversely with the expression level of transfected Flag-cIAP1 (Figures 5A and S4A). However, when overexpressing a mutant cIAP1 (devoid of E3-ubiquitin ligase activity),\textsuperscript{28} Myc-cIAP1 H588A, no change of ULK1 expression was observed (Figure S4B). Next, to unveil mechanisms underline cIAP1-mediated ULK1 expression regulation, we performed ubiquitin analysis in transfected HEK293 cells with an increasing amount of Flag-cIAP1. As expected, the expression of ULK1 in protein level significantly decreased, inversely with the expression level of transfected Flag-cIAP1 (Figures 5A and S4A). However, when overexpressing a mutant cIAP1 (devoid of E3-ubiquitin ligase activity),\textsuperscript{28} Myc-cIAP1 H588A, no change of ULK1 expression was observed (Figure S4B). Next, to unveil mechanisms underline cIAP1-mediated ULK1 expression regulation, we performed ubiquitin analysis in transfected HEK293 cells. Ubiquitinated ULK1 level sharply raised in Flag-cIAP1-overexpression cells, whereas overexpressing Myc-cIAP1 H588A did not change ULK1 ubiquitination (Figure 5B). We next investigate the interaction of cIAP1 with ULK1. We incubated THP-1 cells with MG132 to equal the protein level of ULK1 in shNT, shTRAF2, and shTRAF3 THP-1 cells. Co-IP assay revealed physical association of ULK1 with cIAP1, but the interaction cannot be detected in TRAF2- and TRAF3-knockdown cells (Figure 5C). Interaction of overexpressed cIAP1 with ULK1 can also be detected in HEK293 cells (Figure 5D, lane 2), which significantly increased when co-expressed with TRAF2 and TRAF3 (Figure 5D, lane 5). These data indicated that both TRAF3 and TRAF2 are required for ULK1 and cIAP1 interaction. Interestingly, knockdown of TRAF3 disturbed TRAF2 interaction with ULK1 (Figure 5E), while overexpression of TRAF3 increased interacting activity of TRAF2 and ULK1 (Figure 5F), suggesting that TRAF3 might serve as the primary interacting protein of ULK1 and it to TRAF2.

3.6 | ULK1 participates in LPS/Ng-induced inflammasome

ULK1 participates the very first autophagy-specific complex formation and mediates autophagy initiation.\textsuperscript{29,30} We analyzed the autophagy activation in WT and TRAF3\textsuperscript{MKO} macrophages. We treated WT or TRAF3\textsuperscript{MKO} PEMs with Earle’s Balanced Salt Solution (EBSS), a classical autophagy inducer, and tracking autophagy activation with its marker LC3B and p62. Surprisingly, no significant difference of LC3II and p62 levels could be detected, which suggested TRAF3-deficiency might not result in autophagy activation (Figure 6A). We also treated PEMs with LPS/Ng to observe autophagy marker p62 degradation. However, in spite of
upregulation of ULK1 level, p62 protein level did not decreased under LPS/Ng-treatment (Figure S5).

Recent studies suggest that TRAF3 mediates ubiquitination of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and promotes inflammasome activation.\textsuperscript{20,21} However, it is still largely unknown about TRAF3-mediated inflammasome in macrophages. We examined mature forms of IL-1β p17 and caspase 1 p10 to reveal inflammasome activation in LPS/ATP- and LPS/ Ng-stimulated macrophages. As expected, compared with WT group, a lower level of mature IL-1β p17 and caspase 1 p10 were detected in the supernatant of TRAF3-deficient
macrophages (Figures 6B and S6). Besides LPS/ATP- and LPS/Ng-induced NLRP3 inflammasome, we also evaluated whether TRAF3 is involved in other types of inflammasomes, such as AIM2 and NLRC4. Production of mature IL-1β p17 induced by poly(dA:dT), an AIM2 inflammasome inducer, decreased in TRAF3-deficient BMDMs (Figure S7A). However, TRAF3-deficiency had no influence on inflammasome-formation associated proteins (caspase 1 and IL-1β) in whole-cell lysates and culture medium (supernatant). D-F, TRAF3-deficient BMDMs (TRAF3MKO) were transduced with shNT, shULK1#1, or shULK1#2, respectively. WT, TRAF3MKO shNT, TRAF3MKO shULK1#1, and TRAF3MKO shULK1#2 BMDMs were primed with LPS for 3 hours followed by Ng stimulation for 30 minutes. D, IB analysis of IL-1β p17 and caspase 1 p10 in the supernatant and pro-IL-1β, pro-caspase 1, ULK1, and TRAF3 in whole-cell lysates. E, Cell supernatants were collected and production of IL-1β by BMDMs were examined using mouse IL-1β ELISA kit. F, Supernatants were collected and cell viability was detected by LDH release assay. *P < .05

**FIGURE 6** ULK1 regulates inflammasome activation. A, PEMs were treated with EBSS for indicated times to induce autophagy. IB assays were performed to analyze LC3A, LC3B, p62, and ULK1 protein levels. B, BMDMs were primed with LPS for 3 hours followed by Ng or ATP stimulation for 30 minutes. IB analysis of inflammasome-formation associated proteins (caspase 1 and IL-1β) in whole-cell lysates and culture medium (supernatant). C, THP-1 cells were primed with LPS for 3 hours followed by Ng stimulation for 30 minutes. IB analysis of inflammasome-formation associated proteins (caspase 1 and IL-1β) in whole-cell lysates and culture medium (supernatant). D-F, TRAF3-deficient BMDMs (TRAF3MKO) were transduced with shNT, shULK1#1, or shULK1#2, respectively. WT, TRAF3MKO shNT, TRAF3MKO shULK1#1, and TRAF3MKO shULK1#2 BMDMs were primed with LPS for 3 hours followed by Ng stimulation for 30 minutes. D, IB analysis of IL-1β p17 and caspase 1 p10 in the supernatant and pro-IL-1β, pro-caspase 1, ULK1, and TRAF3 in whole-cell lysates. E, Cell supernatants were collected and production of IL-1β by BMDMs were examined using mouse IL-1β ELISA kit. F, Supernatants were collected and cell viability was detected by LDH release assay. *P < .05

AIM2 inflammasomes but not in NLRC4 inflammasome activation.

Then, control or ULK1 stable knockdown THP-1 cells were treated with LPS/Ng to determine whether ULK1 participates in LPS/Ng-stimulated inflammasome. Interestingly, both precursor and mature forms of caspase 1 and IL-1β were prominently increased in ULK1-deficient cells (Figure 6C), suggesting its considerable role in macrophage inflammasome process. To further determine the function of ULK1 in LPS/Ng-induced inflammasome and macrophage pyroptosis, ULK1 was knocked down in TRAF3-deficient macrophages.
and inflammasome activity and cell death were examined. Compared with TRAF3\textsuperscript{MKO} group, knockdown of ULK1 in TRAF3-deficient BMDMs significantly elevated supernatant mature IL-1β and caspase 1 levels in LPS/Ng-stimulated cells (Figure 6D, lane 6, 9, and 12, Figure 6E), which demonstrated that TRAF3-mediated inflammasome activation is through targeting ULK1. LDH release from macrophages was measured to determine the percentage of pyroptotic cell. As expected, similar to the pattern of IL-1β and caspase 1 activation, LDH release was reduced in TRAF3-deficient BMDMs upon LPS/Ng stimulation (Figure 6F). Meanwhile, compared with TRAF3\textsuperscript{MKO} group, LDH release increased after ULK1 knocked down in TRAF3\textsuperscript{MKO} macrophages (Figure 6F).

Subcellular distribution of ULK1 plays an important role in regulating cell death under adverse environmental or intracellular conditions.\textsuperscript{31,32} The subcellular translocalization of ULK1 in response to LPS/Ng-induced pyroptosis in macrophage is still unclear. Here, we detected nuclear/cytosol and mitochondria/cytosol subcellular distribution of ULK1 in LPS/Ng-stimulated THP-1 cells. LPS/Ng treatment did not change distribution of ULK1 either in nuclear or cytosol fractions (Figure S8A). However, ULK1 is slightly upregulated and translocated to mitochondrial fraction upon pyroptosis inducing by LPS/Ng stimulation (Figure S8B).

### 3.7 LPS/Ng-induced pyroptosis in ULK1-deficient THP-1 cells is dependent on AIF nuclear translocation

To further investigate whether LPS/Ng-induced inflammasome is associated with pyroptotic cell death in ULK1-deficient macrophages, we next examined the cleavage of PARP cleavage and activation of Caspases-3, -7, -8, and -9, a family of protease enzymes playing essential roles in pyroptosis, using immunoblotting. Interestingly, we did not detect cleaved form of PARP in ULK1-deficient THP-1 cells after LPS/Ng treatment (Figure 7A, lane 4 and 6), although LPS/Ng stimulated significant cell death in ULK1-deficient THP-1 cells (Figure 2F). Moreover, active forms of Caspases-3, -7, -8, and -9 could not be observed in LPS/Ng-treated cells (Figure 7A, lane 4 and 6). These results suggested that LPS/Ng-induced pyroptotic cell death in ULK1-deficient THP-1 cells is independent on Caspase-3 cleavage.

Apoptosis inducing factor (AIF) is known to mediate caspase-independent cell death by releasing from mitochondrion into nuclear.\textsuperscript{33} We then detected AIF nuclear translocation by immunoblotting assay. LPS/Ng treatment did not change the concentration of AIF either in nuclear or cytosol (Figure 7B, lane 1 to 4). However, when treated ULK1-deficient THP-1 cells with LPS/Ng for 30 and 60 minutes, AIF distribution in the nuclear obviously increased (Figure 7B, lane 7, 8, 11, and 12). Furthermore, THP-1 cells were double knockdown of both AIF and ULK1 and cell death was examined under LPS/Ng treatment. As shown in Figure 7C, LPS/Ng-induced cell death significantly in ULK1-knockdown THP-1 cells. However, when treated ULK1/AIF double-knockdown THP-1 cells with LPS/Ng, the ratio of cell death reduced (Figure 7C). These results indicated that AIF nuclear translocation play essential role in LPS/Ng triggers cell death in ULK1-deficient THP-1 cells.

### 4 DISCUSSION

Inflammation is a well-known process in many infectious and noninfectious diseases.\textsuperscript{34-36} Mitochondrial dysfunction and amplified ROS production are key events in inflammatory diseases and are responsible for the association between chronic inflammation and increased tumor incidence.\textsuperscript{37,38} Abnormal ROS production promotes inflammation and inflammatory programmed cell death.\textsuperscript{16,22} TNFR-associated factors (TRAFs) are important mediators of inflammatory reactions signaling\textsuperscript{26,39} and ROS regulation.\textsuperscript{40} TRAF6 regulates TLR-stimulated MAPKs and NF-κB activation and is considered important for ROS-dependent activation of innate immune pathways.\textsuperscript{41} However, the role of other TRAF family members in regulating TLR signaling and ROS production is less clear. TRAF3 is known as a critical component of the lymphotixin-β receptor (LTβR) signaling complex and regulates NF-κB signaling.\textsuperscript{42} In the present study, we demonstrated that TRAF3 involves in mitochondrial ROS production in macrophages.

ROS are essential for effective activation of innate immune signaling pathways.\textsuperscript{43} Disrupted mitochondrial membrane potential and ROS generation are often associated with macrophage pyroptosis.\textsuperscript{16} LPS, a TLR4 agonist, treatment together with ATP or nigericin can stimulate inflammasome formation and pyroptotic cell death through activation of caspase 1 and releasing of mature IL-1β, which play essential roles in pathogen defending or stress. The present study demonstrated that LPS/Ng induces inflammasome and pyroptosis in THP-1 and mouse primary macrophages, which significantly decreases in TRAF3-deficient cells.

Interestingly, in the present data, we found that ULK1, a central regulator in autophagy, undergoes a rapid turnover and its protein expression is hardly detected in untreated wild-type BMDMs or PEMs. ULK1 protein levels dramatically increased in TRAF3-deficient macrophages. Recently, evidence showed that Rapamycin treatment reverses increased inflammasome activation in a chronic nonbacterial prostatitis rat model through inducing mTOR/ULK1/ATG13 signaling pathway,\textsuperscript{44} which indicated that autophagy plays essential role in inflammasome activation and pyroptotic cell death. We did not observe increased LC3BII or decreased p62 in either EBSS- or LPS/Ng-induced autophagy
in TRAF3-deficient macrophages compare to wild-type cells. However, ULK1-knockdown resulted in a significant increased ROS level and cell death in LPS/Ng-treated cells. These data indicated that ULK1 may participate in TRAF3-mediated ROS production and pyroptosis in an autophagy-independent manner. LPS/ATP or LPS/Ng-treatment decreases cIAP1 and TRAF3 protein levels and promotes ULK1 accumulation in macrophages. Some evidences have demonstrated that TRAF3 usually forms a ubiquitination complex together with E3 ubiquitin ligases TRAF2 and cIAP1 and regulates downstream target genes, such as NIK, c-Rel, and IRF5. In the present study, we further revealed that TRAF3/TRAF2/cIAP1 form a complex and mediate ULK1 ubiquitination and proteasome-dependent degradation in macrophages (Figure 8).

Mitochondrial dysfunction, inflammasome activation and caspases cleavage are key steps of the pyroptotic cell death. Although we found increased mitochondrial ROS level and activated caspase 1 in ULK1-deficient THP-1 cells, cleaved caspase 3, 7, 8, 9, and PARP cannot be detected in LPS/Ng-treated ULK1-deficient THP-1 cells. Instead, slightly decrease of caspase 3, 7, 8, and 9 protein levels were observed after LPS/Ng stimulation. AIF is known to mediate caspase-independent cell death. The present data demonstrated that LPS/Ng-induced PARP-independent pyroptosis in ULK1-deficient THP-1 cells.

**FIGURE 7** LPS/Ng-induced PARP-independent pyroptosis in ULK1-deficient THP-1 cells. A, THP-1 cells (shNT, shULK1#1, and shULK1#2) were treated with LPS/Ng. PARP and caspases were detected using IB assays. B, THP-1 cells were transduced with shNT or ULK1-specific shRNAs (shULK1#1, shULK1#2). Nuclear and cytosol fractions were extracted, respectively, and IB assays were performed to analyze AIF translocalization. LaminB was detected as a nuclear marker. C, ULK1 knockdown THP-1 cells (shULK1#1) were infected with AIF-specific shRNA as indicated. Cells were treated with LPS/Ng and cell death were detected using SYTOX Red staining (upper). IB analysis of AIF protein levels (lower). *P < .05
Ng treatment induces AIF nuclear translocation, which may play critical roles in inflammasome associated cell death in ULK1-deficient macrophages (Figure 8).

In summary, the present study provided a new understanding of TRAF3-ULK1-mediated pyroptotic cell death in macrophages. TRAF3 plays an essential role in the K48-linked ULK1 ubiquitination. TRAF3 recruits TRAF2 and cIAP to ULK1 and mediated ULK1 degradation. ULK1 undergoes rapid turnover in wild-type macrophages. TRAF3 deficiency blocks ULK1 ubiquitination and results in intracellular ULK1 accumulation. ULK1 inhibits LPS/Ng-induced mitochondrial ROS production and AIF nuclear translocation, which contributes to PARP-independent cell death. Moreover, ULK1 reduces caspase 1 activation and inflammasome formation under LPS/Ng treatment. Interestingly, LPS/Ng treatment inhibits TRAF3/TRAF2/cIAP-mediated ULK1 ubiquitination and promotes ULK1 stabilization, which results in accumulation of ULK1 and might form a negative feedback loop and regulates macrophage inflammasome and pyroptotic cell death.

ACKNOWLEDGMENTS
We thank R. Brink for mutant mice. We also thank H. Zhang, the personnel from Hebei University Animal Facility. This study was supported the Natural Science Foundation of Hebei Province (Grant No. H2019201259), National Natural Science Foundation of China (Grant No. 31301143), and the Hebei Youth Topnotch Talent Support Program.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
Y. Shen, W.-w. Liu, X. Zhang, J.-g. Shi, S. Jiang, L. Zheng, J.-h. Shi performed the experiments and acquired the data, Y. Qin, B. Liu, and J.-h. Shi analyzed and interpreted the data. J.-h. Shi conceived and designed the project and wrote the article.

REFERENCES
1. Korzenik J, Larsen MD, Nielsen J, Kjeldsen J, Norgard BM. Increased risk of developing Crohn's disease or ulcerative colitis in 17 018 patients while under treatment with anti-TNFalpha agents, particularly etanercept, for autoimmune diseases other than inflammatory bowel disease. *Aliment Pharmacol Ther*. 2019;50:289-294.
2. Daskalaki MG, Vyrla D, Harizani M, et al. Neorogioltriol and related diterpenes from the red alga Laurencia inhibit inflammatory...
bowel disease in mice by suppressing M1 and promoting M2-like macrophage responses. Mar Drugs. 2019;17(2):97.

3. Banos-Hernandez CJ, Navarro-Zarza JE, Buscara R, et al. Macrophage migration inhibitory factor polymorphisms are a potential susceptibility marker in systemic sclerosis from southern Mexican population: association with MIF mRNA expression and cytokine profile. Clin Rheumatol. 2019;38:1643-1654.

4. Liu Y, Gao Y, Yang J, Shi C, Wang Y, Xu Y. MicroRNA-381 reduces inflammation and infiltration of macrophages in polyomavirus via downregulating HMG1. Int J Oncol. 2018;53:1332-1342.

5. Stefan-Lifshitz M, Karakose E, Cui L, et al. Epigenetic modulation of beta cells by interferon-alpha via PNPT1/26a/TET2 triggers autoimmune diabetes. JCI Insight. 2019;4:e126663.

6. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496:445-455.

7. Wang J, Sahoo M, Lantier L, et al. Caspase-11-dependent pyroptosis of lung epithelial cells protects from melioidosis while caspase-1 mediates macrophage pyroptosis and production of IL-18. PLoS Pathog. 2018;14:e1007105.

8. Mascarenhas DPA, Zamboni DS. Inflammasome activation in legionella-infected macrophages. Methods Mol Biol. 2019;1921:305-319.

9. Riestra AM, Valderrama JA, Patras KA, et al. Trichomonas vaginalis induces NLRP3 inflammasome activation and pyroptotic cell death in human macrophages. J Innate Immun. 2019;11:86-98.

10. O’Meara TR, Duah K, Guo CX, et al. High-throughput screening identifies genes required for Candida albicans induction of macrophage pyroptosis. mBio. 2018;9:e01581-18.

11. O’Meara TR, Cowen LE. Insights into the host-pathogen interaction: C. albicans manipulation of macrophage pyroptosis. Microbiol cell. 2018;5:566-568.

12. Immanuel CN, Teng B, Dong B, et al. Apoptosis signal-regulating kinase-1 promotes inflammasome priming in macrophages. Am J Physiol Lung Cell Mol Physiol. 2019;316:L418-L427.

13. Li CG, Yan L, Mai FY, et al. Baicalin inhibits NOD-like receptor family, pyrin containing domain 3 inflammasome activation in mouse macrophages. Front Immunol. 2017;8:1409.

14. Miao EA, Rajan JV, Aderem A. Caspase-1-induced pyroptotic cell death. Immunol Rev. 2011;243:206-214.

15. Harris J, Deen N, Zamani S, Hasnat MA. Mitophagy and the release of inflammatory cytokines. Mitochondrion. 2018;41:2-8.

16. Wang Y, Shi P, Chen Q, et al. Mitochondrial ROS promote macrophage pyroptosis by inducing GSDMD oxidation. J Mol Cell Biol. 2019;11(12):1069-1082.

17. Guven-Maierov E, Keskin O, Gursoy A, et al. TRAF3 signaling: competitive binding and evolvability of adaptive viral molecular mimicry. Biochim Biophys Acta. 2016;1860:2646-2655.

18. Zarnegar BJ, Wang Y, Mahoney DJ, et al. Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat Immunol. 2008;9:1371-1378.

19. Jin J, Xiao YC, Hu HB, et al. Proinflammatory TLR signalling is regulated by a TRAF2-dependent proteolysis mechanism in macrophages. Nat Commun. 2015;6:5930.

20. Guan K, Wei C, Zheng Z, et al. MAVS promotes inflammasome activation by targeting ASC for K63-linked ubiquitination via the E3 ligase TRAF3. J Immunol. 2015;194:4880-4890.

21. Siu KL, Yuen KS, Castano-Rodriguez C, et al. Severe acute respiratory syndrome Coronavirus ORF3a protein activates the NLRP3 inflammasome by promoting TRAF3-dependent ubiquitination of ASC. FASEB J. 2019;33(8):8865-8877.

22. Xu M, Wang L, Wang M, et al. Mitochondrial ROS and NLRP3 inflammasome in acute ozone-induced murine model of airway inflammation and bronchial hyperresponsiveness. Free Radiac Res. 2019;53(7):780-790.

23. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J. 2009;417:1-13.

24. Dewaele M, Maes H, Agostinis P. ROS-mediated mechanisms of autophagy stimulation and their relevance in cancer therapy. Autophagy. 2010;6:838-854.

25. Van Erp AC, Hoeksma D, Rebolloleda RA, et al. The crosstalk between ROS and autophagy in the field of transplantation medicine. Oxid Med Cell Longev. 2017;2017:7120962.

26. Shi JH, Sun SC. Tumor necrosis factor receptor-associated factor regulation of nuclear factor kappaB and mitogen-activated protein kinase pathways. Front Immunol. 2018;9:1849.

27. Liao G, Zhang M, Harhaj EW, Sun SC. Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. J Biol Chem. 2004;279:26243-26250.

28. Li X, Yang Y, Ashwell JD, TNF-RII and c-IAPI mediate ubiquitination and degradation of TRAF2. Nature. 2002;416:345-347.

29. Zachari M, Ganley IG. The mammalian ULK1 complex and autophagy initiation. Essays Biochem. 2017;61:585-596.

30. Vargas JNS, Wang CX, Bunker E, et al. Spatiotemporal control of ULK1 activation by NDP52 and TBK1 during selective autophagy. Mol Cell. 2019;74:347-362.e6.

31. Joshi A, Iyengar R, Joo JH, et al. Nuclear ULK1 promotes cell death in response to oxidative stress through PARP1. Cell Death Differ. 2016;23:216-230.

32. Wu W, Tian W, Hu Z, et al. ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy. EMBO Rep. 2014;15:566-575.

33. Delavallee L, Cabon L, Galan-Malo P, Lorenzo HK, Susin SA. AIF-mediated caspase-independent necroptosis: a new chance for targeted therapeutics. Int J BMB Life. 2011;63:221-232.

34. Back M, Yurdagul A Jr, Tabas I, Oorni K, Kovanen PT. Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. Nat Rev Cardiol. 2019;16:389-406.

35. Fujita K, Hayashi T, Matsushita M, Uemura M, Nonomura N. Obesity, inflammation, and prostate cancer. J Clin Med. 2019;8:201.

36. Hine AM, Loke P. Intestinal macrophages in resolving inflammation. J Immunol. 2019;203:593-599.

37. El-Kenawi A, Ruffell B. Inflammation, ROS, and mutagenesis. Cancer Cell. 2017;32:727-729.

38. Canli O, Nicolas AM, Gupta J, et al. Myeloid cell-derived reactive oxygen species induce epithelial mutagenesis. Cancer Discov. 2017;32:869-883.e5.

39. Zong X, Zhao J, Wang H, et al. Mettl3 deficiency sustains long-chain fatty acid absorption through suppressing Traf6-dependent inflammation response. J Immunol. 2019;202:567-578.

40. Ruan H, Zhang Z, Tian L, Wang S, Hu S, Qiao J. The Salmonella effector SopB prevents ROS-induced apoptosis of epithelial cells by retarding TRAF6 recruitment to mitochondria. Biochem Biophys Res Comm. 2016;478:618-623.

41. Matsuzawa A, Saegusa K, Noguchi T, et al. ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. Nat Immunol. 2005;6:587-592.
42. Shi JH, Sun SC. Tumor necrosis factor receptor-associated factor regulation of nuclear factor kappa B and mitogen-activated protein kinase pathways. *Front Immunol*. 2018;9:1849.

43. Adam C, Wohlfarth J, Haussmann M, et al. Allergy-inducing chromium compounds trigger potent innate immune stimulation via ROS-dependent inflammasome activation. *J Invest Dermatol*. 2017;137:367-376.

44. Su Y, Lu J, Chen X, et al. Rapamycin alleviates hormone imbalance-induced chronic nonbacterial inflammation in rat prostate through activating autophagy via the mTOR/ULK1/ATG13 signaling pathway. *Inflammation*. 2018;41:1384-1395.

45. Vallabhapurapu S, Matsuzawa A, Zhang W, et al. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. *Nat Immunol*. 2008;9:1364-1370.

---

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Shen Y, Liu W-W, Zhang X, et al. TRAF3 promotes ROS production and pyroptosis by targeting ULK1 ubiquitination in macrophages. *The FASEB Journal*. 2020;34:7144–7159. [https://doi.org/10.1096/fj.201903073R](https://doi.org/10.1096/fj.201903073R)