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TRIM50 promotes NLRP3 inflammasome activation by directly inducing NLRP3 oligomerization

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Abstract

Tripartite motif protein (TRIM) 50 is a new member of the tripartite motif family, and its biological function and the molecular mechanism it is involved in remain largely unknown. The NOD-like receptor family protein (NLRP)3 inflammasome is actively involved in a wide array of biological processes while mechanisms of its regulation remain to be fully clarified. Here, we demonstrate the role of TRIM50 in NLRP3 inflammasome activation. In contrast to the conventional E3 ligase functions of TRIM proteins, TRIM50 mediates direct oligomerization of NLRP3, thereby suppressing its ubiquitination and promoting inflammasome activation. Mechanistically, TRIM50 directly interacts with NLRP3 through its RING domain and induces NLRP3 oligomerization via its coiled-coil domain. Finally, we show that TRIM50 promotes NLRP3 inflammasome-mediated diseases in mice. We thus reveal a novel regulatory mechanism of NLRP3 via TRIM50 and suggest that modulating TRIM50 might represent a therapeutic strategy for NLRP3-dependent pathologies.

Keywords inflammation; NLRP3 inflammasome; oligomerization; TRIM50; ubiquitination

Subject Categories Immunology; Post-translational Modifications & Proteolysis; Signal Transduction

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Introduction

The NOD-like receptor family protein (NLRP)3 inflammasome is a multiprotein complex capable of sensing cellular stress derived from a wide variety of stimuli, including invading pathogens, pore-forming toxins, endogenous danger signals, and metabolic dysfunctions (Schroder *et al*, 2010; Broz & Dixit, 2016; Ising *et al*, 2019). The activation of NLRP3 inflammasome involves two consecutive steps, the priming step triggered by cytokines or pathogen-associated molecular patterns (PAMPs) to mediate the upregulation of NLRP3 and IL-1β transcription, and the activation step leading to the assembly of NLRP3 inflammasome via the self-oligomerization and the recruitment of apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and pro-caspase-1. Activation of NLRP3 inflammasome facilitates the secretion of pro-inflammatory cytokines IL-1β and IL-18, leading to the induction of robust inflammation and pyroptosis (Kayagaki *et al*, 2015; Shi *et al*, 2015; Kesavardhana & Kanneganti, 2017; Ising *et al*, 2019).

The NLRP3 inflammasome plays a critical role in the host defenses against pathogens, whereas its dysregulation has also been linked to the pathogenesis of several inflammatory disorders (Goldberg *et al*, 2017; Ising *et al*, 2019; Sharma & Kanneganti, 2021). Therefore, the molecular mechanism of NLRP3 inflammasome activation has been extensively investigated, and accumulating evidence indicates that the post-translational modification, including ubiquitination, phosphorylation, and sumoylation, participates in the regulation of NLRP3 inflammasome by directly modifying NLRP3 (Song *et al*, 2016; Spalinger *et al*, 2017; Barry *et al*, 2018). Though the regulation of NLRP3 inflammasome activation has been extensively investigated, its exact molecular regulatory mechanism remained to be fully clarified.

The tripartite motif-containing (TRIM) family members are a series of proteins with a wide range of biological activities, and they usually contain several featured structures including a really interesting new gene (RING) domain, a B-box domain, and a coiled-coil domain (Di Rienzo *et al*, 2020). Most of the TRIM family members are E3 ubiquitin (Ub) ligases, and they exert their biological effects by inducing the ubiquitination modification of target proteins (Hatakeyama, 2017). The role of TRIM proteins in the regulation of NLRP3 inflammasome activation and innate immunity has attracted much research interest in recent years (Hu *et al*, 2016; Song *et al*, 2016). TRIM50 is a newly identified member of the TRIM family, and the currently limited investigations suggested it as a typical E3 ubiquitin ligase in Williams–Beuren syndrome, ovarian cancer, and hepatocellular carcinoma (Micale *et al*, 2008; Ma *et al*, 2018; Qiu *et al*, 2019). It is recognized that NLRP3 is heavily ubiquitinated

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under basal conditions, and ubiquitination plays a pivotal role in regulating NLRP3 inflammasome (Py *et al*, 2013). The currently defined effect of TRIM50 is ubiquitination modification through its RING domain (Micale *et al*, 2008), indicating a possible involvement of TRIM50 in the regulation of NLRP3 inflammasome activation. However, the biological function of TRIM50 in the innate immune response remains unknown and here we reveal that TRIM50 positively regulates the NLRP3 inflammasome in a way different from its conventional E3 ligase function.

In this study, we have investigated the role of TRIM50 in the process of NLRP3 inflammasome activation. We show that TRIM50 directly interacts with NLRP3 via its RING domain, which results in the abrogation of its RING-type ubiquitination ligase activity, and further leads to NLRP3 oligomerization and NLRP3 inflammasome activation. These data indicate that TRIM50 plays an important regulatory role in NLRP3 inflammasome activation, and also suggest a potential therapeutic strategy by targeting TRIM50 in a variety of NLRP3 inflammasome-involved diseases.

Results

TRIM50 directly interacts with NLRP3

TRIM50 is a recently identified new member of TRIM family and whether it is involved in the regulation of NLRP3 inflammasome activation is not known. To define the role of TRIM50 in NLRP3 inflammasome activation, we did immunoprecipitation (IP) assays to verify whether TRIM50 interacts with NLRP3. Exogenous IP in HEK293T cells and endogenous IP in mouse peritoneal macrophages both indicated that TRIM50 interacted with NLRP3 (Fig 1A,B). Then, we expressed TRIM50 and NLRP3 protein using an *in vitro* transcription and translation system, and the IP assay showed that TRIM50 directly interacted with NLRP3 *in vitro* (Fig 1C). Confocal microscopy further revealed that TRIM50 and NLRP3 co-localized in the cytosol (Fig EV1A). Further investigation showed that TRIM50 only interacted with NLRP3 while there was no interaction with either ASC or Caspase 1 (Fig EV1B). After the NLRP3 inflammasome was activated, the interaction between TRIM50 and NLRP3 significantly increased in a time-dependent manner (Fig 1D), which indicated that the interaction between TRIM50 and NLRP3 was involved in NLRP3 inflammasome activation. Endogenous IP also verified the involvement of the interaction between TRIM50 and NLRP3 in the activation process of the NLRP3 inflammasome (Fig 1E). The following confocal data revealed specific staining of TRIM50 and NLRP3 co-localization in the cytosol of human THP1 cells and mouse peritoneal macrophages, and their interaction was significantly enhanced after LPS and ATP stimulation (Figs 1F and EV1C), indicating the efficient interaction between NLRP3 and TRIM50 during NLRP3 inflammasome activation. These data demonstrate that TRIM50 and NLRP3 are *bona fide* interacting partners in macrophages and that their

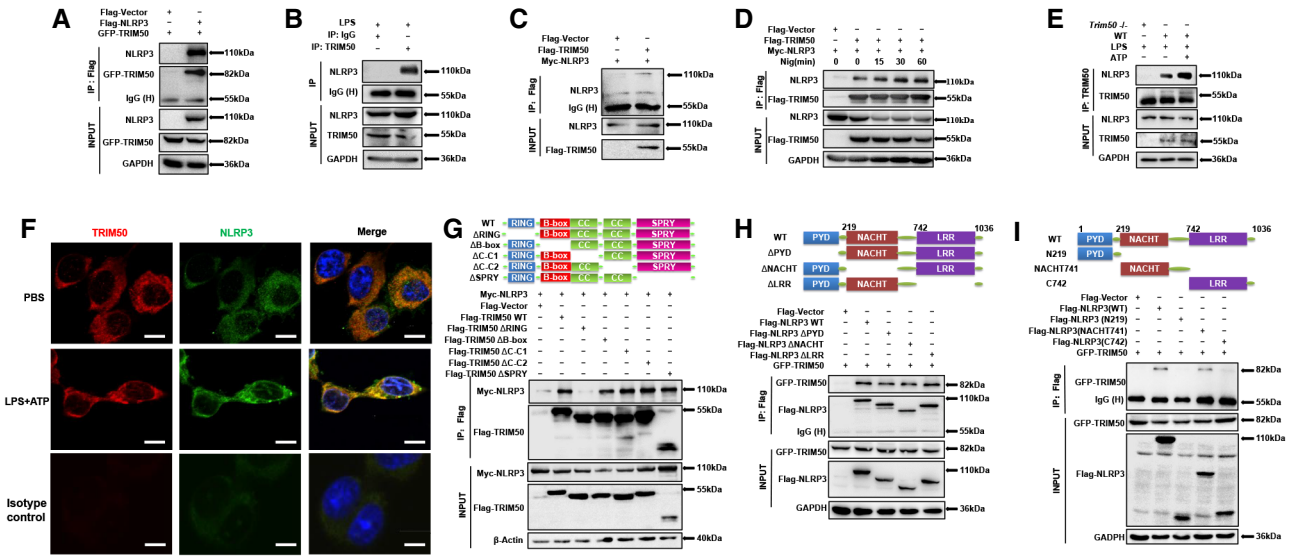

Figure 1. TRIM50 directly interacts with NLRP3.

A Exogenous immunoprecipitation assay of the interaction between TRIM50 and NLRP3.

B Endogenous immunoprecipitation assay of the interaction between TRIM50 and NLRP3 in mouse peritoneal macrophages.

C TRIM50 and NLRP3 proteins were expressed and obtained from the *in vitro* translation system, and their interactions were further analyzed.

D Co-IP assay of the interaction between NLRP3 and TRIM50 after stimulation with nigericin for 0, 15, 30, and 60 min.

E Co-IP assay of the interaction between NLRP3 and TRIM50 in mouse peritoneal macrophages primed with LPS for 6 h and treated with ATP for 30 min.

F Confocal microscopy assay of the interaction between TRIM50 and NLRP3 in THP1 cells with stimulation by LPS and ATP. Scale bars: 10 μm.

G Schematic diagram of human TRIM50 (WT) and its truncation mutants was presented(top), and the interaction of TRIM50 (TRIM50 truncation mutants) and NLRP3 was detected by IP assay.

H, I Schematic diagram of human NLRP3 (WT) and its truncation mutants (top), and immunoprecipitation assay of the interaction of NLRP3 (NLRP3 truncation mutants) with TRIM50 in HEK293T cells.

Data information: Data are representative of three biological replicates with similar results.
Source data are available online for this figure.

interaction significantly increases during NLRP3 inflammasome activation.

To further determine the interaction domains between TRIM50 and NLRP3, we constructed a series of domain-truncated mutants of these two proteins. TRIM50 has a RING-finger domain, a B-box domain, two coiled-coil domains, and a SPRY domain; while NLRP3 has an N-terminal PYD domain, a NACHT domain, and a C-terminal LRR domain. IP assays revealed that the interaction between TRIM50 and NLRP3 was abolished when the RING domain of TRIM50 was deleted (Fig 1G), which indicated that TRIM50 interacted with NLRP3 through its RING domain. However, all of the domain-deleted NLRP3 mutants were still competent for the interaction with TRIM50 (Fig 1H), thus we further constructed a series of constructs of NLRP3 overexpressing each domain. IP assay then showed that the NACHT741 region, the connection area between NACHT and LRR domain, was the direct interacting domain with NLRP3 (Fig 1I). These data indicate that the NACHT741 region of NLRP3 and the RING domain of TRIM50 represent the molecular basis for their direct interaction. Taken together, these data indicate that TRIM50 directly interacts with NLRP3 and that their interaction is enhanced during NLRP3 inflammasome activation.

TRIM50 promotes NLRP3 inflammasome activation

To define whether the direct interaction between TRIM50 and NLRP3 has any effect on NLRP3 inflammasome activation, we knocked down *Trim50* in mouse peritoneal macrophages by specific small interfering RNA against *Trim50* (Fig EV2A,B). When we stimulated these macrophages with LPS, a typical stimulator commonly used for the priming of the NLRP3 inflammasome (Ising *et al*, 2019), the data showed that knockdown of *Trim50* dramatically reduced the levels of NLRP3 but had no significant effect on the protein levels of ASC, caspase-1, and IL-1β (Fig 2A). Further investigation showed that knockdown of *Trim50* had no influence on the mRNA levels of *Nlrp3* and *Il-1β* in these LPS-primed mouse peritoneal macrophages (Fig 2B). These data indicate that TRIM50 has positive regulatory effects on NLRP3.

The NLRP3 protein is the rate-limiting factor for NLRP3 inflammasome activation (Huai *et al*, 2014; Huang *et al*, 2019; Deng *et al*, 2022). Thus, we were interested in defining whether the positive regulation of NLRP3 by TRIM50 might have any effect on NLRP3 inflammasome activation. The data in peritoneal macrophages showed that after knockdown of *Trim50*, the cleavage of caspase-1

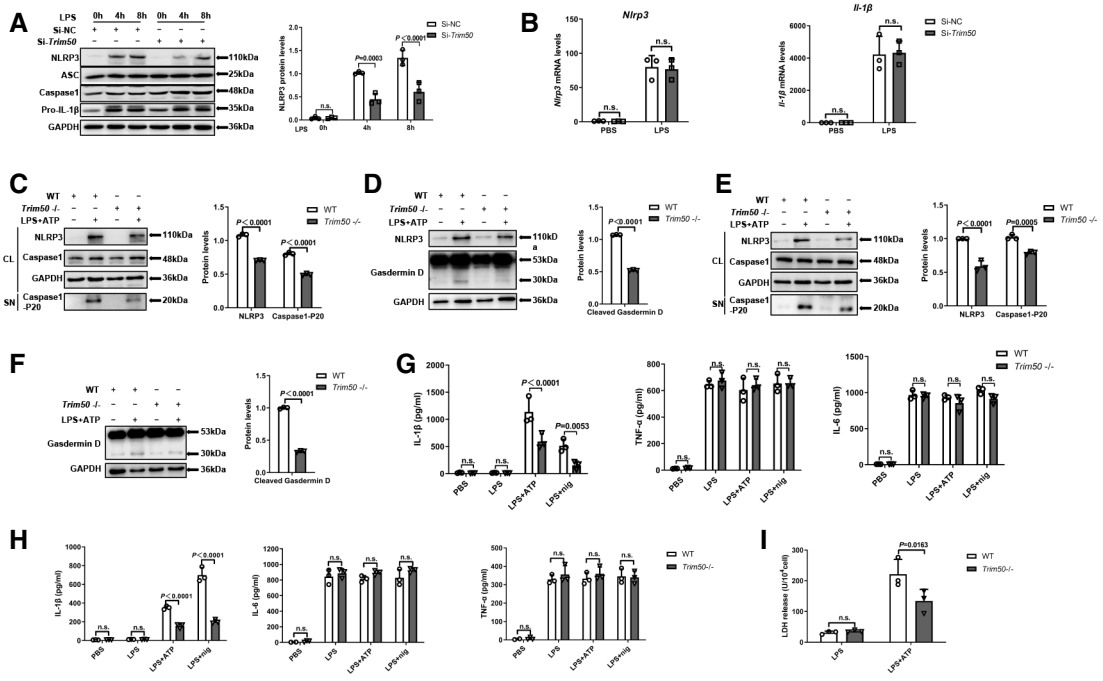

Figure 2. TRIM50 promotes NLRP3 inflammasome activation.

A Immunoblot analysis of the NLRP3 protein level in the mouse peritoneal macrophages transfected with Si-*Trim50*.

B qRT–PCR analysis of *Nlrp3* and *IL-1β* mRNA levels in LPS-primed Si-*Trim50* transfected mouse peritoneal macrophages.

C, D Immunoblot analysis of Caspase1-P20 (C) and Gasdermin D (D) of WT or *Trim50*$^{-/-}$ mouse peritoneal macrophages after LPS stimulation for 6 h and ATP treatment for 30 min.

E, F Immunoblot analysis of Caspase1-P20 (E) and Gasdermin D (F) of WT or *Trim50*$^{-/-}$ BMDM cells after LPS stimulation for 6 h and ATP treatment for 30 min.

G, H ELISA analysis of IL-1β, TNF-α, and IL-6 in the supernatant of WT or *Trim50*$^{-/-}$ peritoneal macrophages and BMDM cells with the activation of NLRP3 inflammasome. $n = 3$.

I LDH analysis of WT or *Trim50*$^{-/-}$ mouse peritoneal macrophages with the activation of NLRP3 inflammasome. $n = 3$.

Data information: Band densities were quantitated by "Image J" software and normalized to GAPDH. *P*-values are shown, two-tailed Student's *t*-test (A, C–F) or two-way analysis of variance (ANOVA) (B, G, H, and I). Data are representative of three biological replicates with similar results (mean ± SD in A–I).
Source data are available online for this figure.

and Gasdermin D was significantly inhibited in ATP-stimulated macrophages (Fig EV2C,D), which indicated that TRIM50 could promote NLRP3 inflammasome activation. When we knocked down *TRIM50* in human monocytic THP1 cells by siRNA, the protein levels of NLRP3 were significantly decreased (Fig EV2E), and the production of IL-1β was also significantly inhibited (Fig EV2F). We further isolated peritoneal macrophages and BMDMs from *Trim50*$^{-/-}$ mice, and the data verified that cleavage of Caspase1 and Gasdermin D were significantly decreased in both *Trim50*$^{-/-}$ peritoneal macrophages (Fig 2C,D) and *Trim50*$^{-/-}$ BMDM cells (Fig 2E,F) compared with their wild-type counterparts. ELISA assays further showed that *Trim50* deficiency significantly reduced IL-1β secretion in both ATP or nigericin stimulated peritoneal macrophages and BMDM cells, while no effect on the secretion of TNF-α or IL-6 was observed (Fig 2G,H). In addition, we detected the release of lactate dehydrogenase (LDH) by macrophages, and the stimulated *Trim50*$^{-/-}$ cells showed less LDH release compared with their WT counterparts (Fig 2I). Caspase 11, as a cytosolic LPS receptor, interacts with NLRP3 and potentiates the noncanonical activation of the NLRP3 inflammasome (Moretti *et al*, 2022). Thus, we further tried to define the role of TRIM50 in the noncanonical NLRP3 inflammasome activation pathway in LPS-transfected macrophages. The data showed that deletion of *Trim50* had no significant effect on caspase 11 activation, while the cytosolic LPS-induced cleavage of Caspase 1 and Gasdermin D was significantly decreased in *Trim50*$^{-/-}$ macrophages (Fig EV2G,H). In summary, these data indicate that TRIM50 could positively regulate the NLRP3 inflammasome activation by upregulating the protein levels of NLRP3 in macrophages.

TRIM50 reduces the ubiquitination of NLRP3

TRIM50 is a RING-type E3 ubiquitin ligase. Our data demonstrated that TRIM50 directly interacted with NLRP3 via its RING domain, raising the question how exactly TRIM50 affects NLRP3 ubiquitination. We analyzed the ubiquitination of NLRP3 after the exogenous overexpression of TRIM50, and the data revealed that TRIM50 failed to put ubiquitin chains into NLRP3. By contrast, NLRP3 ubiquitination was significantly attenuated by TRIM50 overexpression (Fig 3A), and further investigations showed that all of the tested ubiquitin types, including K6, K11, K27, K29, K33, K48, and K63 linked ubiquitination of NLRP3, were all significantly suppressed by TRIM50 (Fig EV3A), which verified that TRIM50 negatively regulates NLRP3 ubiquitination. Further investigation showed that the self-ubiquitination of TRIM50 was significantly abrogated by the interaction between TRIM50 and NLRP3 (Fig EV3B), which indicated the blockade of the functional TRIM50 RING domain by its interaction with NLRP3. When we did the ubiquitination assay in *Trim50*-deleted macrophages, we saw that the attenuation of NLRP3 ubiquitination was almost completely abolished (Fig 3B), which further verified TRIM50-mediated attenuation of NLRP3 ubiquitination. BRCC3 is a reported deubiquitinase of NLRP3 (Py *et al*, 2013), and our data further showed that

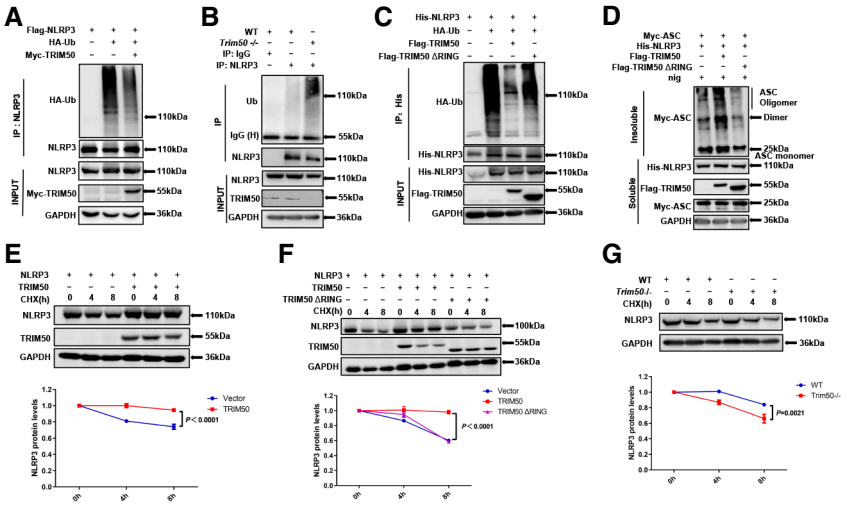

Figure 3. TRIM50 reduces the ubiquitination of NLRP3.

A Immunoprecipitation analysis of the NLRP3 ubiquitination in HEK293T cells transfected with HA-ubiquitin, His-NLRP3, and Flag-TRIM50 plasmids.

B Immunoprecipitation analysis of the endogenous NLRP3 ubiquitination in WT or *Trim50*$^{-/-}$ BMDM cells.

C Immunoprecipitation analysis of NLRP3 ubiquitination in HEK293T cells transfected with NLRP3, HA-ubiquitin, TRIM50, or TRIM50 (ΔRING) mutant plasmids.

D Immunoblot analysis of the ASC oligomerization in cross-linked cytosolic pellets (insoluble) and the whole cell lysate (soluble) in HEK293T cells with nigericin treatment for 30 min.

E Immunoblot analysis of NLRP3 protein levels in cycloheximide (CHX) treated HEK293T cells.

F Immunoblot analysis of NLRP3 in HEK293T cells transfected with NLRP3 plasmid and TRIM50 or TRIM50 (ΔRING) mutant, followed by further treatment with CHX for 0, 4, or 8 h.

G Immunoblot analysis of NLRP3 in mouse peritoneal macrophages, followed by further treatment with CHX for 0, 4, or 8 h.

Data information: Band densities were quantitated by "Image J" software and normalized to GAPDH. *P*-values are shown, two-way ANOVA (E–G). Data are representative of three biological replicates with similar results (mean ± SD in E–G).
Source data are available online for this figure.

TRIM50 and BRCC3 interacted with each other (Fig EV3C,D), indicating a possible involvement of BRCC3 in TRIM50-regulated NLRP3 inflammasome activation. Altogether, these data demonstrate that instead of the conventional role of inducing ubiquitination, TRIM50 significantly suppressed the ubiquitination of NLRP3, probably with the involvement of BRCC3.

The RING domain of TRIM50 is the interaction domain between NLRP3 and TRIM50, and we further tried to define the role of the RING domain regarding the ubiquitination status of NLRP3. Our data showed that a RING domain-deleted TRIM50 mutant almost completely abolished the attenuation of ubiquitination induced by TRIM50 (Fig 3C). ASC oligomerization is a required step in NLRP3 inflammasome activation (Cai *et al*, 2014; Lu *et al*, 2014), and our data showed that the RING domain-deleted mutant also abolished TRIM50-induced ASC oligomerization (Fig 3D), indicating that the RING domain was required for TRIM50 to exert its suppressive effect on NLRP3 ubiquitination. Next, we constructed an enzyme dead mutant of TRIM50 (C53A) by replacing cysteine with alanine at position 53 within the RING domain. The self-ubiquitination of TRIM50 was almost completely abolished after the transfection of the TRIM50 (C53A) mutant, indicating that the catalytic activity of this mutant was successfully abolished (Fig EV3E). Further investigation showed that the TRIM50 (C53A) mutant abrogated the interaction between TRIM50 and NLRP3 (Fig EV3F), and TRIM50-mediated suppression of NLRP3 ubiquitination was also significantly reduced by TRIM50 (C53A) mutant (Fig EV3G), which indicated that the enzymatic active C53 residue is pivotal for the RING domain-mediated interaction between TRIM50 and NLRP3. Altogether these data indicate that the RING domain-mediated interaction between TRIM50 and NLRP3 is pivotal for the TRIM50-induced attenuation of NLRP3 ubiquitination.

So far, we have described the negative regulation of NLRP3 ubiquitination by TRIM50. Thus, we further tried to define whether this effect influences the protein stability of NLRP3. When *de novo* protein synthesis was blocked by cycloheximide (CHX), the degradation of NLRP3 was significantly suppressed in TRIM50-transfected cells (Fig 3E), which indicated that TRIM50 maintained the stability of NLRP3. Further investigations showed that the RING domain-deleted mutant of TRIM50 almost completely abolished NLRP3 protein stability maintained by TRIM50 (Fig 3F), which validated the functional role of the RING domain. When we did protein stability assays in *Trim50*-deleted macrophages, the data revealed that the degradation of NLRP3 was significantly enhanced in *Trim50*$^{-/-}$ macrophages (Fig 3G). CHX assays further showed that TRIM50 had no effect on the protein stability of NLRC4 and AIM2, the key proteins in other inflammasomes (Fig EV3H), and NLRC4 and AIM2 inflammasome activation was not affected by TRIM50 either (Fig EV3I). Thus, these data indicate that TRIM50 regulates NLRP3 inflammasomes by its effect on NLRP3. Altogether, these data indicate that TRIM50 prevents the ubiquitination of NLRP3 and maintains the stability of the protein.

TRIM50 promotes NLRP3 inflammasome activation by inducing NLRP3 oligomerization

We have demonstrated that TRIM50 facilitates NLRP3 inflammasome activation. It is recognized that NLRP3 oligomerization is a required step for recruiting ASC and inducing NLRP3 inflammasome activation (Swanson *et al*, 2019). Thus, we further tried to define whether TRIM50 regulates NLRP3 oligomerization. Semi-denaturing detergent agarose-gel electrophoresis (SDD–AGE) assays showed that TRIM50 could significantly promote the oligomerization of NLRP3 (Fig 4A), and the levels of NLRP3 oligomerization and ASC oligomerization were both significantly enhanced by TRIM50 after the NLRP3 inflammasome was activated (Fig 4B,C), further underlining the involvement of TRIM50 in NLRP3 inflammasome activation. The most recent reports showed that NLRP3 activation is associated with defined post-translational modifications (Swanson *et al*, 2019), while our data showed that TRIM50 did not exert its effect via sumoylation or tyrosine phosphorylation (Fig EV4A–D). Further investigation showed that the oligomerization of NLRP3 and ASC were significantly reduced in both *Trim50*$^{-/-}$ peritoneal macrophages and BMDM cells (Fig 4D–G); while the RING domain-deleted TRIM50 mutant almost completely abrogated TRIM50-induced NLRP3 oligomerization (Fig 4H), indicating a critical involvement of TRIM50 in the induction of NLRP3 oligomerization.

So far, we have defined roles for TRIM50 for the oligomerization and the attenuation of NLRP3 ubiquitination. We were further interested in defining the direct effect of TRIM50 on NLRP3. We constructed an *in vitro* oligomerization system and detected the effect of TRIM50 on the oligomerization of NLRP3. The data showed that TRIM50 could directly induce NLRP3 oligomerization *in vitro* (Fig 4I). Tranilast and MCC950 are recognized potent NLRP3 inhibitors (Yang *et al*, 2019). Tranilast directly binds to NLRP3 and inhibits NLRP3-NLRP3 interactions to prevent NLRP3-dependent inflammasome diseases, and MCC950 blocks NLRP3-induced ASC oligomerization by inhibiting NLRP3-ASC interactions (Coll *et al*, 2015; Huang *et al*, 2018). To further clarify the working mechanism of NLRP3, we treated cells with tranilast and MCC950 and then detected the effect of TRIM50 on NLRP3 ubiquitination. The data showed that the suppressive effect on NLRP3 ubiquitination induced by TRIM50 was significantly rescued by tranilast but not MCC950 (Fig 4J), indicating that TRIM50 directly exerted its effect at the level of NLRP3-NLRP3 interactions and the attenuation of ubiquitination was a subsequent indirect consequence. To further clarify the effect of TRIM50-induced NLRP3 oligomerization on NLRP3 inflammasome activation, we investigated the possible involvement of NEK7, a protein that directly promotes NLRP3 oligomerization by interacting with NLRP3 (Shi *et al*, 2016). The IP assay indicated that TRIM50 interacted with NEK7 (Fig EV5A) and NEK7 significantly promoted the interaction between TRIM50 and NLRP3 (Fig EV5B). To further define the contribution of NEK7 to TRIM50-induced NLRP3 oligomerization, we blocked NEK7 by its specific inhibitor Licochalcone B (Li *et al*, 2022). The data showed that compared with the WT control, both deletion of *Trim50* and inhibition of NEK7 significantly decreased NLRP3 oligomerization (Fig EV5C), indicating the involvement of both TRIM50 and NEK7 in NLRP3 inflammasome activation. Altogether, these data indicate that TRIM50 directly induces NLRP3 oligomerization and NEK7 promotes the interaction between TRIM50 and NLRP3, thus leading to NLRP3 inflammasome activation.

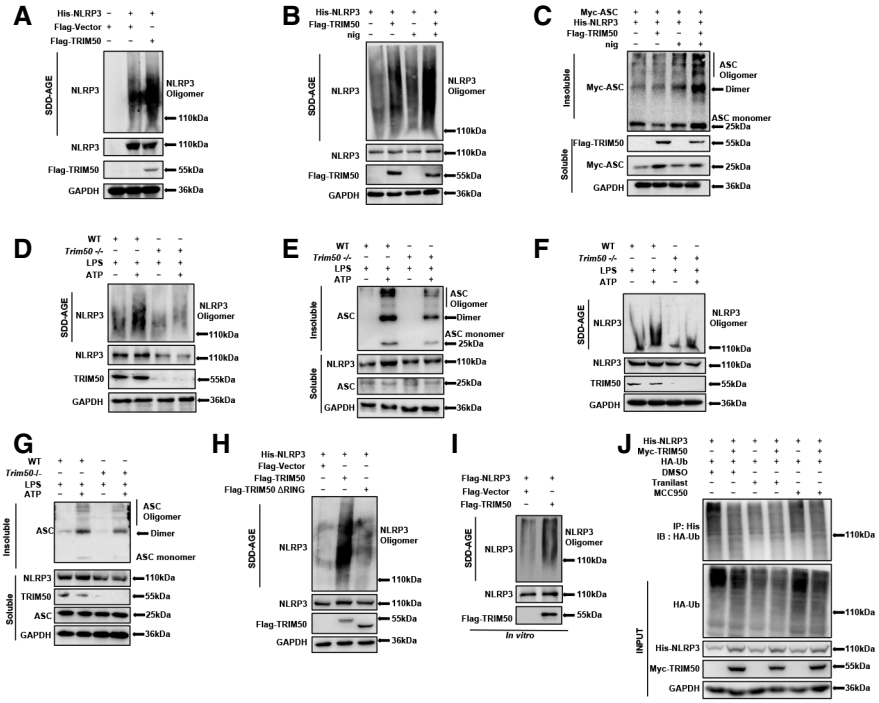

Figure 4. TRIM50 promotes NLRP3 inflammasome activation by inducing NLRP3 oligomerization.

A SDD–AGE analysis of NLRP3 oligomerization in HEK293T cells transfected with NLRP3 and TRIM50 plasmids.

B SDD–AGE analysis of NLRP3 oligomerization in NLRP3 and TRIM50 transfected HEK293T cells followed by nigericin treatment for 30 min.

C Immunoblot analysis of ASC oligomerization in cross-linked cytosolic pellets (insoluble) and whole cell lysate (soluble) in HEK293T cells transfected with NLRP3, ASC, and TRIM50 plasmids followed by nigericin treatment for 30 min.

D SDD–AGE analysis of NLRP3 oligomerization in WT or *Trim50*$^{-/-}$ peritoneal macrophages with LPS treatment for 6 h and ATP treatment for 30 min.

E Immunoblot analysis of ASC oligomerization in cross-linked cytosolic pellets (insoluble) and whole cell lysate (soluble) in WT or *Trim50*$^{-/-}$ peritoneal macrophages by LPS stimulation for 6 h and ATP treatment for 30 min.

F SDD–AGE analysis of NLRP3 oligomerization in WT or *Trim50*$^{-/-}$ BMDM cells with LPS treatment for 6 h and ATP treatment for 30 min.

G Immunoblot analysis of ASC oligomerization in cross-linked cytosolic pellets (insoluble) and whole cell lysate (soluble) in WT or *Trim50*$^{-/-}$ BMDM cells by LPS stimulation for 6 h and ATP treatment for 30 min.

H SDD–AGE analysis of the oligomerization of NLRP3 in HEK293T cells transfected with His-NLRP3 and TRIM50 or TRIM50(ΔRING) mutant.

I *In vitro* NLRP3 oligomerization assay with *in vitro* translated TRIM50 and NLRP3 proteins.

J Immunoprecipitation analysis of NLRP3 ubiquitination in HA-ubiquitin, His-NLRP3, and Myc-TRIM50 transfected HEK293T cells with further treatment with tranilast or MCC950 for 24 h.

Data information: Data are representative of three biological replicates with similar results.
Source data are available online for this figure.

TRIM50 mediates NLRP3 oligomerization via its dual coiled-coil domain

We have demonstrated that TRIM50 directly induced NLRP3 oligomerization, leading to the reduction in NLRP3 ubiquitination and NLRP3 inflammasome activation. Thus, we were further interested in clarifying which domain of TRIM50 is responsible for the regulation of NLRP3. We transfected a series of domain-truncated TRIM50 mutants together with HA-ubiquitin plasmids, and the following ubiquitination assays showed that both the RING domain-deleted and the coiled-coil domain-deleted mutants of TRIM50 could significantly rescue the reduction in NLRP3 ubiquitination induced by TRIM50 (Fig 5A), which indicates the involvement of these domains in the TRIM50-mediated effect on NLRP3. Coiled-coil domains are recognized to induce oligomerization of multiple proteins (Burkhard *et al*, 2001), and our data further shows that the coiled-coil domain-deleted TRIM50 mutants (ΔC−C1 and ΔC−C2) could significantly reduce the NLRP3 oligomerization (Fig 5B), while both mutants could induce TRIM50 oligomerization (Fig 5C), indicating distinct roles for the sole and dual coiled-coil domains of TRIM50. Further investigations showed that after transfection with both coiled-coil domain-deleted mutants of TRIM50 (TRIM50 ΔC−C1/2), the oligomerization of NLRP3 and TRIM50 were both significantly abolished (Fig 5D). These data indicate that the presence of the dual coiled-coil domain of TRIM50 was required for NLRP3 oligomerization, while the single coiled-coil domain induces TRIM50 self-oligomerization.

When we blocked *de novo* protein synthesis by CHX, we found that NLRP3 protein stability maintained by TRIM50 was significantly rescued by the coiled-coil domain-deleted mutants (ΔC−C1 and ΔC−C2) of TRIM50 (Fig 5E), and ASC oligomerization was also rescued (Fig 5F), which further verified the involvement of the coiled-coil domains of TRIM50 in the induction of NLRP3 inflammasome activation. Altogether, these data indicate that the unique dual

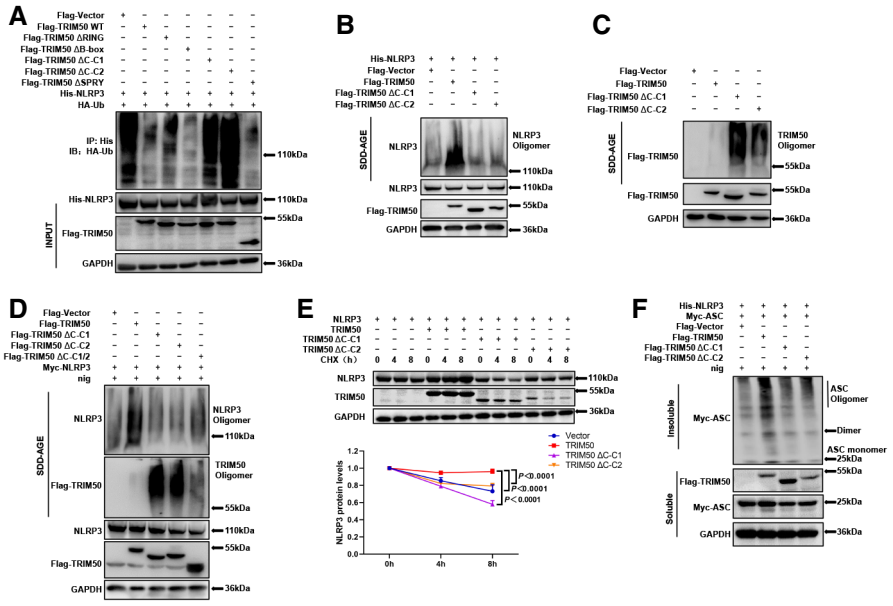

Figure 5. The coiled-coil domains of TRIM50 are required for the regulation of the NLRP3 inflammasome.

A Immunoprecipitation analysis of NLRP3 ubiquitination in HEK293T cells transfected with NLRP3, HA-ubiquitin, TRIM50, or its truncation mutants.

B SDD–AGE analysis of NLRP3 oligomerization in NLRP3 together with TRIM50 or its coiled-coil domain-deleted mutants (ΔC−C1 and ΔC−C2) transfected HEK293T cells.

C SDD–AGE analysis of the oligomerization of TRIM50 in TRIM50 or its coiled-coil domain-deleted mutants (ΔC−C1 and ΔC−C2) transfected HEK293T cells.

D SDD–AGE analysis of the oligomerization of NLRP3 and TRIM50 in HEK293T cells overexpressing NLRP3 together with TRIM50 or its coiled-coil domain-deleted mutants.

E Immunoblot analysis of NLRP3 in HEK293T cells transfected with NLRP3 plasmid and TRIM50 coiled-coil domain-deleted mutants, followed by the cycloheximide (CHX) treatment for 0, 4, or 8 h.

F Immunoblot analysis of ASC oligomerization in cross-linked cytosolic pellets (insoluble) and whole cell lysate(soluble) in HEK293T cells transfected with His-NLRP3 and TRIM50 or TRIM50 coiled-coil domain-deleted mutants.

Data information: Band densities were quantitated by "Image J" software and normalized to GAPDH. *P*-values are shown, two-way ANOVA(E). Data are representative of three biological replicates with similar results (mean ± SD in E).
Source data are available online for this figure.

coiled-coil domain characteristics of TRIM50 are required for NLRP3 oligomerization and NLRP3 inflammasome activation.

TRIM50 is involved in NLRP3 inflammasome-mediated diseases in mice

In a final set of experiments, we tried to demonstrate the role of TRIM50 *in vivo*. First, we constructed an LPS-induced endotoxic shock model by intraperitoneal injection of LPS in mice. Further investigations showed that the overall survival time of LPS-challenged *Trim50*$^{-/-}$ mice was significantly prolonged compared with wild-type mice (Fig 6A). The serum level of IL-1β in *Trim50* knockout mice was significantly decreased compared to that of their wild-type counterpart, whereas *Trim50* deficiency had no significant effect on the serum levels of TNF-α and IL-6 (Fig 6B), indicating the specific regulation of NLRP3 inflammasome by TRIM50. In addition, hematoxylin and eosin (H&E) staining of the lung tissue showed that *Trim50* deficient mice had less septal mononuclear cells and lymphocyte infiltration, lower levels of alveolar edema, and lower acute lung injury (ALI) scores (Fig 6C), which indicates that *Trim50* deficiency significantly ameliorated NLRP3-mediated inflammation and tissue damage *in vivo*. An alum-induced acute peritonitis model also showed that the number of neutrophils and monocytes infiltrating the peritoneal lavage of *Trim50* knockout mice was significantly lower than in wild-type mice (Fig 6D), which further proves a positive regulatory role of TRIM50 in NLRP3 inflammasome involved diseases.

We further investigated the regulatory effect of TRIM50 on NLRP3 inflammasome in a folic acid (FA)-induced acute tubular necrosis (ATN) model by intraperitoneal injection of FA in mice. Thirty-six hours after the injection, the mice were sacrificed and the data showed that the level of NLRP3 in the kidney was significantly lower in *Trim50*$^{-/-}$ mice, and the cleavage of pro-Caspase 1 and pro-IL-1β were also significantly decreased in *Trim50*$^{-/-}$ mice compared with wild-type mice (Fig 6E). H&E staining and periodic acid–Schiff (PAS) staining showed that renal inflammation and edema were both significantly ameliorated in *Trim50*$^{-/-}$ mice compared with their wild-type counterparts (Fig 6F,G). These data indicate that the effect of TRIM50 on NLRP3 inflammasome activation significantly contributes to the progression of NLRP3 inflammasome-mediated diseases.

Altogether, these data demonstrate that TRIM50 directly interacts with NLRP3 via its RING domain and induces NLRP3 oligomerization and attenuates NLRP3 ubiquitination, thus leading to NLRP3

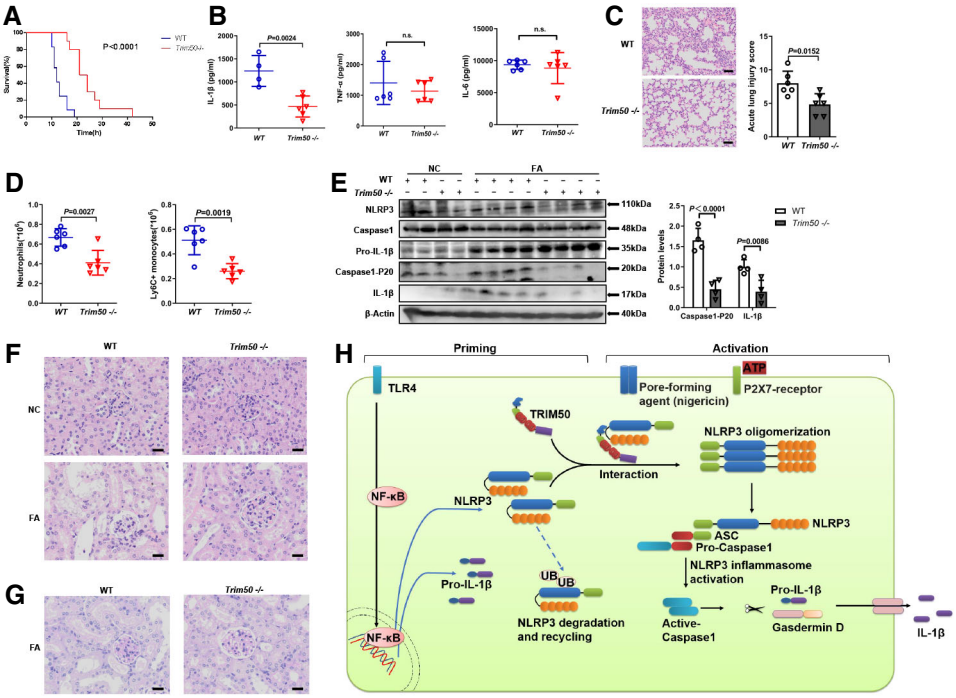

Figure 6. TRIM50 regulates NLRP3 inflammasome-related diseases in mice.

A Survival status of WT and *Trim50*$^{-/-}$ mice after intraperitoneal injection with LPS (n = 10 mice/group).

B ELISA analysis of IL-1β, TNF-α, and IL-6 in serum of WT or Trim50$^{-/-}$ mice after i.p. injection with LPS for 6 h (n = 4–6 mice/group).

C Histopathological analysis of the lung tissues by hematoxylin and eosin (H&E) staining. The quantified lung injury was depicted by defined clinical parameters in ALI score (n = 6 mice/group). Scale bars: 50 μm.

D Flow cytometry analysis of the neutrophils (left) and Ly6C+ monocytes (right) in the peritoneum of WT or *Trim50*$^{-/-}$ mice after i.p. injection with Alum (n = 6 mice/group).

E WT or *Trim50*$^{-/-}$ mice were i.p. injected with FA for 36 h, followed by immunoblot analysis of NLRP3, pro-IL-1β, Caspase1 p20, and IL-1β in the kidney tissue samples.

F H&E staining of kidney tissue sections. Scale bars: 20 μm.

G Periodic acid-Schiff (PAS) staining of kidney tissue sections. Scale bars: 20 μm.

H Schematic diagram of the regulatory role of TRIM50 in NLRP3 inflammasome activation.

Data information: *P*-values are shown, two-way ANOVA (A) or two-tailed Student's *t*-test (B–E). Data are representative of three biological replicates with similar results (mean ± SD in A–E).
Source data are available online for this figure.

inflammasome activation (Fig 6H). This study demonstrates that TRIM50 plays a critical role in NLRP3-dependent diseases and suggests potential therapeutic strategies for these inflammatory diseases by targeting TRIM50.

Discussion

NLRP3 is an innate immune sensor that can trigger an inflammatory cascade in response to multiple intracellular and extracellular stresses. Though most innate immune sensors have limited specificity for pathogen-associated molecular patterns or damage-associated molecular patterns, NLRP3 has the unique characteristics of being activated by a wide variety of unrelated stimuli, suggesting that NLRP3 senses common cellular stress signals instead of undergoing direct interactions with all these triggers (Swanson *et al*, 2019; Bai *et al*, 2020). Thus, the activation of NLRP3 inflammasomes must be tightly regulated to ensure that it can effectively exert its effect while avoiding excessive inflammatory responses. The investigation of the regulatory mechanism of NLRP3 inflammasome activation has been dramatically developed in recent years, and the most recent studies showing NEK7-mediated NLRP3 inflammasome activation addressed the pivotal role of NLRP3 oligomerization in inducing NLRP3 inflammasome activation (Shi *et al*, 2016; Chen *et al*, 2019). However, how NLRP3 oligomerization is dynamically regulated remained to be fully understood. The role of TRIM50 in NLRP3 oligomerization and NLRP3 inflammasome activation is reported here for the first time.

TRIM50 belongs to the tripartite motif protein family and TRIM proteins constitute one of the largest subfamilies of RING-type E3 ligases that induce ubiquitination to further regulate multiple physiological processes (Popovic *et al*, 2014). The currently recognized function of TRIM50 is ubiquitination via its RING domain (Micale *et al*, 2008; Ma *et al*, 2018), and the RING domain is a well-defined catalytic unit of TRIMs (Kiss *et al*, 2021). However, our study unexpectedly demonstrates that TRIM50 directly interacts with NLRP3 via its RING domain, thus bringing up the intriguing question how TRIM50 promotes NLRP3 inflammasome activation independent of

its ubiquitination function. Here we demonstrate that in contrary to the conventional RING-type E3 ligase activity of TRIM proteins, TRIM50 dramatically inhibits the ubiquitination of NLRP3 while inducing NLRP3 oligomerization and NLRP3 inflammasome activation. Our further investigations revealed that the oligomerization of NLRP3 is a direct effect induced by TRIM50, while the reduction in NLRP3 ubiquitination is an indirect effect, probably caused by the involvement of the deubiquitinase BRCC3. The precise regulatory role of TRIM50 in NLRP3 deubiquitination remains to be fully clarified in the future.

Consistent with our study showing oligomerization induced by TRIM50, other TRIM proteins including TRIM31, TRIM65, and TRIM13 have also been reported to induce the oligomerization of target proteins (Lang *et al*, 2017; Liu *et al*, 2017; Ji *et al*, 2020). More effects other than E3 ligase activities have been reported for other TRIM proteins. For example, TRIM38 induces sumoylation, TRIM14 recruits USP14 and promotes deubiquitination, TRIM23 exerts ADP ribosylation factor (ARF) GTPase activity to promote autophagy, and TRIM32 interacts with and mediates the degradation of TRIF in an E3 ligase activity-independent manner (Chen *et al*, 2016; Hu *et al*, 2016; Sparrer *et al*, 2017; Yang *et al*, 2017). These reports suggested that TRIM proteins have versatile biological activities aside from their roles as E3 ligases, which further expands the research and application fields of TRIM families. Here, we demonstrate for the first time that TRIM50 interacts with NLRP3 via its RING domain and further mediates the oligomerization of NLRP3 and NLRP3 inflammasome activation.

We further demonstrate that TRIM50 mediates the oligomerization of NLRP3 through its coiled-coil domain. Coiled-coil domains, as ubiquitous structural protein motifs found in about 10% of all eukaryotic proteins, constitute the principal oligomerization motifs in proteins to promote the formation of homo- and hetero-oligomers (Burkhard *et al*, 2001). This domain can also be involved in signal transduction and help to maintain the mechanical stability of the cell (Herrmann & Aebi, 2004). The coiled-coil domain is one of the featured structures of TRIM proteins and it facilitates the formation of antiparallel dimers and the construction of higher-order assemblies (Koliopoulos *et al*, 2016). Thus, self-associations via the coiled-coil domain have been suggested to be crucial for the catalytic activity of TRIMs, although its mechanism remains to be clarified (Koliopoulos *et al*, 2016). In contrast to most TRIM proteins that usually contain the sole coiled-coil domain, TRIM50 contains dual coiled-coil domains, indicating the possibility of its unique function based on these structures. Here, we demonstrate that TRIM50 does not self-oligomerize while it induces NLRP3 oligomerization because of the presence of the dual coiled-coil domains. Deletion of either or both of these two coiled-coil domains of TRIM50 abrogates NLRP3 oligomerization, indicating that the unique dual coiled-coil region of TRIM50 is required for this effect on NLRP3. Though coiled-coil domains have been extensively investigated in recent years (Diao *et al*, 2015; Hsu *et al*, 2020; Lu *et al*, 2020), the role of the dual coiled-coil domain of TRIM50 in inducing NLRP3 oligomerization and promoting NLRP3 inflammasome activation was reported here for the first time. Since it was recently recognized that target therapy based on the coiled-coil domain may be a promising clinical application, our data suggest a novel therapeutic strategy for NLRP3-dependent diseases by modulating the coiled-coil domain in TRIM50.

In recent years, aberrant activation of the NLRP3 inflammasome has been implicated in various diseases including cardiovascular, diabetes, and neurodegenerative diseases (Goldberg *et al*, 2017; Ising *et al*, 2019; Sharma & Kanneganti, 2021), which elicits a tremendous interest in exploring potential inhibitors of NLRP3 inflammasomes to restrain tissue destructions. In the past decade, great efforts were taken to develop small molecule inhibitors for NLRP3 inflammasomes and some of them have shown remarkable therapeutic potential (Coll *et al*, 2015; Huang *et al*, 2018; Yang *et al*, 2019; Li *et al*, 2022). However, we are still very far from successful clinical applications for these inhibitors. Thus, the development of novel efficient inhibitors for NLRP3 inflammasome is of great significance. Our study indicates a therapeutic strategy by precisely targeting the key functional domain of TRIM50, which might be applied for the clinical treatment of NLRP3-dependent diseases.

In this study, we define a novel role of TRIM50 in the positive regulation of NLRP3 inflammasome activation. We demonstrate that TRIM50 directly interacts with NLRP3 via its RING domain and abolishes the E3 ligase activity of TRIM50. We further demonstrate that the interaction between TRIM50 and NLRP3 induces the oligomerization of NLRP3 via the coiled-coil domain of TRIM50, leading to the activation of the NLRP3 inflammasome. Thus, this study uncovered a novel regulatory mechanism of NLRP3 inflammasome activation and proposes TRIM50 as a novel therapeutic target for NLRP3 inflammasome-associated diseases.

Materials and Methods

Cell culture and transfections

HEK293T cells were cultured in DMEM (R10-013-CV, Corning, USA) and human monocytic THP1 cells were cultured in 1640 medium (10-040-CV, Corning, USA). The culture medium was supplemented with 10% FBS (S711-001S, LONSERA, Uruguay), penicillin (100 U/ml), and streptomycin (100 μg/ml). All the cells were free from mycoplasma and were authenticated. To obtain mouse primary peritoneal macrophages, C57BL/6J mice (male, 6–8 weeks old) were intraperitoneally injected with 6% starch broth. Peritoneal exudate cells (PECs) were harvested 72 h after the injection, and further cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Adherent monolayer cells were harvested as peritoneal macrophages 4 h after the attachment. For the induction of primary BMDMs, cells were isolated from mouse bone marrow and cultured in prepared culture media (20% FBS, 30% L929 cells supernatant, 50% DMEM, penicillin, and streptomycin as mentioned above).

Human and mouse genes specific siRNA were synthesized by Sigma-Aldrich (St. Louis, USA), and cells were transfected with plasmids or small interference RNA according to the previously described procedure (Zhang *et al*, 2014). TRIM50 plasmid was synthesized by Origene (Maryland, USA), and NLRP3, ASC, and Caspase1 plasmids were got as previously described (Wei *et al*, 2015). NEK7 and BRCC3 plasmids were synthesized by Miaolingbio (Wuhan, China). TRIM50 mutants and NLRP3 mutants were constructed by using a site-directed mutagenesis kit (TOYOBO Life Science, Osaka, Japan) according to the manufacturer's instructions. Gene-specific siRNA were listed as follows: Ctr-siRNA sense:

UUCUCCGAACGUGUCACGUTT, and antisense: ACGUGACACGUUCGGAGAATT; mouse *Trim50*-siRNA sense: GCUUGCAGGCCGCAUAUCUdTdT, and antisense: AGAUAUGCGGCCUGCAAGCdTdT; human *TRIM50*-siRNA1 sense: GGCUCUACCUGCACUAUGAdTdT, and antisense: UCAUAGUGCAGGUAGAGCCdTdT; human *TRIM50*-siRNA2 sense: CCCUUAGAAGGCGCAUUCAdTdT, and antisense: UGAAUGCGCCUUCUAAGGGdTdT; human *TRIM50*-siRNA3 sense: CACCACAAGUUCAUCCGGAdTdT, and antisense: UCCGGAUGAACUUGUGGUGdTdT.

Mice

C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). *Trim50* KO mice on C57BL/6J background were generated by Cyagen Biosciences Inc. (Guangzhou, China) using CRISPR-Pro technology. All mice were housed in a specific pathogen-free facility in the Laboratory Animal Center of Shandong University in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All of the mice experiments were approved by the Scientific Investigation Board of the Medical School of Shandong University.

Reagents and antibodies

LPS (*Escherichia coli*, 0111: B4, L4130), ATP (A6419), nigericin (N7143), folic acid (F7876), anti-Flag antibody(F1804), and small interference RNAs targeting TRIM50 were bought from Sigma-Aldrich (St. Louis, USA). Flagellin and poly (dA:dT) were from Invivogen (San Diego, CA). Disuccinimidyl suberate (DSS, D155694) was bought from Aladdin (Shanghai, China). The antibody including anti-NLRP3 (AG-20B-0014), anti-Caspase1 p20 (AG-20B-0042), anti-ASC (AG-25B-0006) were from Adipogen (San Diego, CA, USA). The anti-GAPDH (60004-1-Ig), anti-β-actin (66009-1-Ig), anti-HA (51064-2-AP), anti-GFP (66002-1-Ig), anti-His (66005-1-Ig) antibodies were from Proteintech (Chicago, USA). The anti-IL-1β (12242S), anti-Gasdermin D (39754S) and anti-phospho-tyrosine (8954S) antibodies were from Cell Signaling Technology (Danfoss, USA). The anti-Caspase 11 antibody (ab180673) was from Abcam (Cambridge, UK). The anti-TRIM50 (TA335359) and anti-Myc (TA150054) antibodies were from OriGene (Maryland, USA). Protein A/G PLUS-Agarose(sc-2003) used for IP and anti-ubiquitin (sc-8017) antibodies were from Santa Cruz Biotechnology (California, USA). The anti-TRIM50 antibody (orb1652) was from Biorbyt Ltd. (Cambridge, UK). CHX, MCC950, and Tranilast were bought from Selleck (Houston, USA). Licochalcone B was from MCE (State of New Jersey, USA).

Western blot and immunofluorescence assay

Western blot and immunofluorescence microscopy assays were performed and evaluated as previously described (Zhang *et al*, 2014; Wei *et al*, 2015; Guo *et al*, 2018). Cell culture supernatants were harvested and concentrated for immunoblotting with Amicon Ultra10 K (UFC5010) from Millipore (Massachusetts, USA).

Co-IP assay

For coimmunoprecipitation assays, cells were harvested and further lysed in 400 μl IP buffer (50 mM Tri-HCl pH7.4; 150 mM NaCl; 1% NP-40; 5 mM EDTA). Cell lysates were collected after centrifugation at 14,000 *g* for 20 min and further incubated overnight with protein A/G Plus-Agarose together with a specific antibody. 4×10^6 of HEK293T cells and 1×10^7 of macrophages were harvested for ubiquitination or other PTM assay. Cell lysates were added with 2% SDS and boiled at 100°C for 15 min to denature the protein. The supernatants were diluted with IP buffer to the solution with < 0.1% SDS before the IP assay was performed. Then, the beads were washed 5 times with IP buffer and eluted by boiling with 1× SDS sample buffer.

Real-time quantitative PCR

Total RNA was prepared with Trizol reagent according to the manufacturer's instructions. The RNA was reverse transcribed using a PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A) from TAKARA (Dajin, Japan). Real-time PCR was performed using FastSYBR Mixture (CW0659s) from CWBio (Jiangsu, China). Gene-specific primers were listed as follows: mouse *Nlrp3* forwards: ATTACCCGCCCGAGAAAGG, and reverse: TCGCAGCAAAGATCCACACAG; mouse *Trim50* forwards: CCCATTTGCCTGGAGGTCTTC, and reverse: CAGGACAGCATAGC TCGGAG; mouse *β-Actin* forwards: GGCTGTATTCCCCTCCATCG, and reverse: CCAGTTGGTAACAATGCCATGT.

In vitro binding assays

Myc-NLRP3 and Flag-TRIM50 proteins were expressed with a TNT Quick Coupled Transcription/Translation System (Promega) according to the instructions of the manufacturer. Binding assays were followed by IP.

NLRP3 oligomerization and ASC oligomerization assays

For NLRP3 oligomerization assay, the whole cell extracts were lysed and centrifuged at 14,000 *g* for 10 min. Forty microliter of the supernatant was used as input for SDS–PAGE experiments, and the rest of the supernatant was used for SDD–AGE assay according to the published protocol (Liu *et al*, 2017). For ASC oligomerization assay, cells were lysed in Triton X-100 lysis buffer. The supernatant of the cell lysate was taken as the soluble component, and the precipitate was taken as the insoluble component. The precipitate was further washed and re-suspended with PBS, and cross-linking treated with 2 mM disuccinimidyl suberate (DSS) at 37°C for 30 min. The samples were further analyzed for protein oligomerization.

ELISA and LDH assays

Mouse IL-1β, TNF-α, and IL-6 were detected by ELISA kits (Dakewe Biotech Company Ltd., Shenzhen, China), and human IL-1β was detected by ELISA kit (Elabscience, Wuhan, China) according to the manufacturer's instructions. LDH detection kit was bought from Beyotime (BC0685, Beijing, China) and LDH assay was performed according to the manufacturer's instructions.

Animal models

Sex-matched C57/BL6 mice and $Trim50^{-/-}$ mice (6–8 weeks old) were used for the construction of animal models in this study. The

mice were randomly assigned, and the background of mice is blinded to the major investigator. For LPS-induced endotoxic shock, mice were i.p. injected with 30 mg/kg of LPS. Six hours after the injection, mice were sacrificed and the levels of IL-1β, TNF-α, and IL-6 in the sera were measured by ELISA. The lung tissues of mice were dissected and fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Histological analysis of the lung tissue was performed according to the published protocol (Magupalli *et al*, 2020). For the survival assay, mice were intraperitoneally injected with 20 mg/kg LPS and observed for up to 48 h to monitor their survival status. For Alum-induced peritonitis, mice were intraperitoneally injected with 800 μg of alum before the mice were sacrificed for further investigation 12 h after the injection. The peritoneal cavities were washed to obtain the peritoneal exudate cells for the flow cytometry assay. For folic acid-induced acute tubular necrosis (ATN), mice were intraperitoneally injected with 250 mg/kg of folic acid. The mice were sacrificed 36 h after the injection and the kidneys were then dissected. Kidney tissues were ground and disrupted for immunoblot analysis, and the remaining kidney tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS), and examined by light microscopy for histologic changes.

Statistical analyses

Data were statistically analyzed using GraphPad Prism software (GraphPad, CA, USA). Statistical significance among groups was evaluated with a two-tailed Student's *t*-test or two-way analysis of variance. P value < 0.05 (two-tailed) was considered statistically significant, and the data were presented as mean ± SD.

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

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Author contributions
Yueke Lin: Data curation; software; validation; investigation; visualization; methodology; writing – original draft. **Xiaoting Lv:** Investigation; methodology. **Caiyu Sun:** Investigation. **Yanlin Sun:** Methodology. **Min Yang:** Methodology. **Dapeng Ma:** Methodology. **Weiqiang Jing:** Methodology. **Yunxue Zhao:** Methodology. **Yeping Cheng:** Methodology. **Haocheng Xuan:** Methodology. **Lihui Han:** Conceptualization; resources; supervision; funding acquisition; project administration; writing – review and editing.

Disclosure and competing interests statement
The authors declare that they have no conflict of interest.

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