

A20/Nrdp1 interaction alters the inflammatory signaling profile by mediating K48- and K63-linked polyubiquitination of effectors MyD88 and TBK1

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A20 is a potent anti-inflammatory protein that mediates both inflammation and ubiquitination in mammals, but the related mechanisms are not clear. In this study, we performed mass spectrometry (MS) screening, gene ontology (GO) analysis, and coimmunoprecipitation (co-IP) in a lipopolysaccharide (LPS)-induced inflammatory cell model to identify novel A20-interacting proteins. We confirmed that the E3 ubiquitin ligase Nrdp1, also known as ring finger protein 41 (RNF41), interacted with A20 in LPS-stimulated cells. Further co-IP analysis demonstrated that when A20 was knocked out, degradation-inducing K48-linked ubiquitination of inflammatory effector MyD88 was decreased, but protein interaction-mediated K63-linked ubiquitination of another inflammatory effector TBK1 was increased. Moreover, western blot experiments showed that A20 inhibition induced an increase in levels of MyD88 and phosphorylation of downstream effector proteins as well as of TBK1 and a downstream effector, while Nrdp1 inhibition induced an increase in MyD88 but a decrease in TBK1 levels. When A20 and Nrdp1 were coinhibited, no further change in MyD88 was observed, but TBK1 levels were significantly decreased compared with those upon A20 inhibition alone. Gain- and loss-of-function analyses revealed that the ZnF4 domain of A20 is required for Nrdp1 polyubiquitination. Upon LPS stimulation, the inhibition of Nrdp1 alone increased the secretion of IL-6 and TNF- α but decreased IFN- β secretion, as observed in other studies, suggesting that Nrdp1 preferentially promotes the production of IFN- β . Taken together, these results demonstrated that A20/Nrdp1 interaction is important for A20 anti-inflammation, thus revealing a novel mechanism for the anti-inflammatory effects of A20.

A20 (also named TNFAIP3), a potent anti-inflammatory protein, is involved in various human diseases. Genome-wide association studies have confirmed that polymorphisms in the A20 gene are risk alleles for a range of inflammatory and autoimmune pathologies, including systemic lupus erythematosus, Crohn's disease, and other diseases (1, 2). A20 is also a

tumor suppressor in several types of B cell cancer, including Hodgkin's lymphoma and diffuse large B cell lymphoma (3). Notably, A20-knockout (KO) mice die prematurely due to severe multiorgan inflammation. Mice with tissue-specific deletion of A20 also demonstrate spontaneous or induced inflammatory phenotypes (4, 5). These results suggest that A20 plays an important role in controlling inflammation and acts as a crucial gatekeeper that preserves tissue homeostasis in mammals.

Active A20 has been found to be tightly linked to ubiquitylation. Structurally, mammalian A20 consists of an ovarian tumor (OTU) domain at its N-terminus with deubiquitination (DUB) activity that has been reported to specifically catalyze the hydrolysis of K48- and K63-linked ubiquitin chains. In addition, the seven continuous zinc finger (ZnF) domains at the C-terminus of A20 promote protein interactions and self-oligomerization and have been described mainly in the context of ubiquitin binding, with the ZnF4 domain having the highest affinity for K63-linked ubiquitin and the ZnF7 domain preferentially binding M1 chains (6).

The ubiquitination-editing function of A20 has been shown to negatively regulate NF- κ B signaling by replacing K63-linked chains on receptor-interacting serine/threonine protein kinase 1 (RIPK1) with K48-linked chains, targeting RIPK1 for proteasomal degradation (7). In addition to regulating TNF-induced NF- κ B signaling, A20 has been shown to regulate Toll-like receptor 4 (TLR4)-induced NF- κ B activation through its DUB activity by removing K63-linked chains from tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) and RIPK2 (8). Furthermore, A20 has been shown to negatively regulate T-cell receptor (TCR)-induced NF- κ B activation by deubiquitinating mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) to prevent the interaction between MALT1 and the IKK complex (9). In addition to functioning as a DUB, A20 has been proposed to additionally and/or alternatively inhibit NF- κ B signaling by inducing K48 ubiquitylation-dependent degradation of specific E2 enzymes, thereby affecting the assembly and functionality of E2-E3 ubiquitin complexes, which promote the activation of NF- κ B (10). These findings have confirmed that A20 inhibits

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NF- κ B signaling in a variety of ways based on its unique structural characteristics.

A previous review summarized some of the signaling pathways controlled by A20 through its interactions with specific substrates (11). For example, A20-binding inhibitor of NF- κ B (ABIN1) is recruited by A20 to remove poly-ubiquitinated chains from IKK γ (12). Tax1-binding protein 1 (TAX1BP1) negatively regulates NF- κ B signaling by recruiting A20 to TRAF6 and RIPK1 (13). Itch and RING finger protein 11 (RNF11) have been shown to inhibit NF- κ B signaling as a subunit of an A20 ubiquitin-editing complex (14), which suggests that some ubiquitination substrates can be recruited by A20 to regulate the inflammation signaling pathway.

While there is much evidence that A20 inhibits the inflammatory response by regulating NF- κ B, some studies have demonstrated that A20 can interact with two I κ B kinase-like kinases, IKKI and IKKe, leading to the suppression of interferon (IFN) stimulation response element- and IFN- β promoter-dependent transcription. A20 also regulates other cellular signaling circuits, such as the mTOR signaling pathway, Wnt pathway, and autophagic response (15–17). Furthermore, A20 protects cells from death independently of NF- κ B regulation, and recent work has supported the notion that the protective role of A20 does not primarily rely on its catalytic activities. These findings have shed new light on A20 biology (18). Therefore, it is necessary to characterize the substrate profile of A20 to discover new substrates and molecular mechanisms of A20.

In this study, coimmunoprecipitation (co-IP) coupled with mass spectrometry (MS) was used to screen proteins that interact with A20 after stimulation with inflammatory factors. Through this approach, E3 ubiquitin-protein ligase neuregulin receptor degradation protein-1 (Nrdp1) was discovered to be a new ubiquitination substrate of A20. A20 can promote the interaction between Nrdp1 and myeloid differentiation primary response protein (MyD88), leading to the degradation of MyD88. On the other hand, A20 inhibits the activation of a serine/threonine protein kinase (TBK1) mediated by Nrdp1 and inhibits the secretion of IFN- β . These results reveal a new mechanism by which A20 regulates inflammation through binding with Nrdp1.

Results

Screening for potential A20-binding proteins by co-IP/MS

Prior studies have demonstrated that endogenous subunits of the A20 ubiquitin-editing complex interact in a manner dependent on stimulation by proinflammatory cytokines such as tumor necrosis factor (TNF) or interleukin 1 beta (IL-1 β) or LPS (19). To investigate the mechanism of inflammatory regulation by A20, we examined A20 protein–protein interactions in RAW264.7 cells stimulated with LPS by co-IP coupled with LC-MS/MS. The base peak mass spectrum showed that peaks from the protein mixtures of both the LPS and control groups were well separated, and a total of 9625 proteins were identified in this experiment (Fig. 1A and Table S1). Based on previously reported threshold settings

(20, 21), the distribution of the log₂ ratio (LPS/control) was symmetrical and could be normalized to a Gaussian curve with a mean of 2.23 and an SD of 0.16 (Fig. 1B). Proteins represented by discrete points that were inconsistent with the normal distribution were regarded as differentially expressed proteins (DEPs). In this manner, we identified 313 upregulated DEPs; these proteins were considered candidates for interaction with A20 after LPS stimulation (Fig. 1B). These potential A20-interacting proteins and A20 itself were subjected to Gene Ontology (GO) enrichment analysis. The proteins in the signaling pathway that contains A20 were mainly enriched for the protein binding, zinc ion, and ubiquitin ligase activity terms (Fig. 1C). In addition, analysis of protein structure enrichment showed that the ZnF domain was the most important feature of the A20-binding proteins (Fig. 1D). Therefore, we screened proteins that both contained ZnF domains and exhibited ubiquitin ligase activity (Fig. 1E) and compared their expression levels to evaluate the possibility that they bind A20. Among the potential interacting proteins, Nrdp1 ranked first (Fig. 1F), and its representative mass spectrum characteristics are shown in Figure 1G. A literature search and comparison revealed that Nrdp1 acts as an E3 ubiquitin-protein ligase and regulates the degradation of target proteins (22). However, an interaction between Nrdp1 and A20 has not been reported before.

A20 interacts with Nrdp1 in macrophages

Next, A20 gene KO cells were used to verify the specificity of the interaction between A20 and Nrdp1. In this study, we designed and synthesized four sgRNAs that specifically recognize the A20 gene based on the principle of CRISPR/Cas9 targeted primers (Fig. 2A). The results showed that the KO efficiencies of the sgRNA sequences differed, but the fourth sequence (KO4) completely inhibited expression of the A20 gene (Fig. 2, B and C). Next, the interaction between A20 and Nrdp1 was confirmed to terminate in LPS-induced A20^{+/+} and A20^{-/-} RAW264.7 cells in which the A20 gene had been knocked out (Fig. 2D). In addition, the expression of Nrdp1 and A20 peaked after 60 min of LPS stimulation and then declined. Therefore, we compared expression changes in the Nrdp1 signaling pathway after A20 KO based on a stimulation time of 60 min in subsequent experiments. Moreover, there was no significant difference in the expression of Nrdp1 when A20 was knocked out (Fig. 2, E–G), suggesting that Nrdp1 expression was not affected by A20.

A20 promotes the binding of Nrdp1 and MyD88 while reducing the interaction with TBK1

We next investigated whether A20 participates in the Nrdp1-mediated inflammatory signaling pathway. According to previous research, Nrdp1, as an E3 ubiquitin ligase, inhibits proinflammatory cytokine production by suppressing the MyD88-dependent NF- κ B pathway while increasing IFN- β production by promoting activation of the kinase TBK1 (23). To test the effects of A20 on Nrdp1-related pathways, we first detected the effects of A20 on expression of the downstream

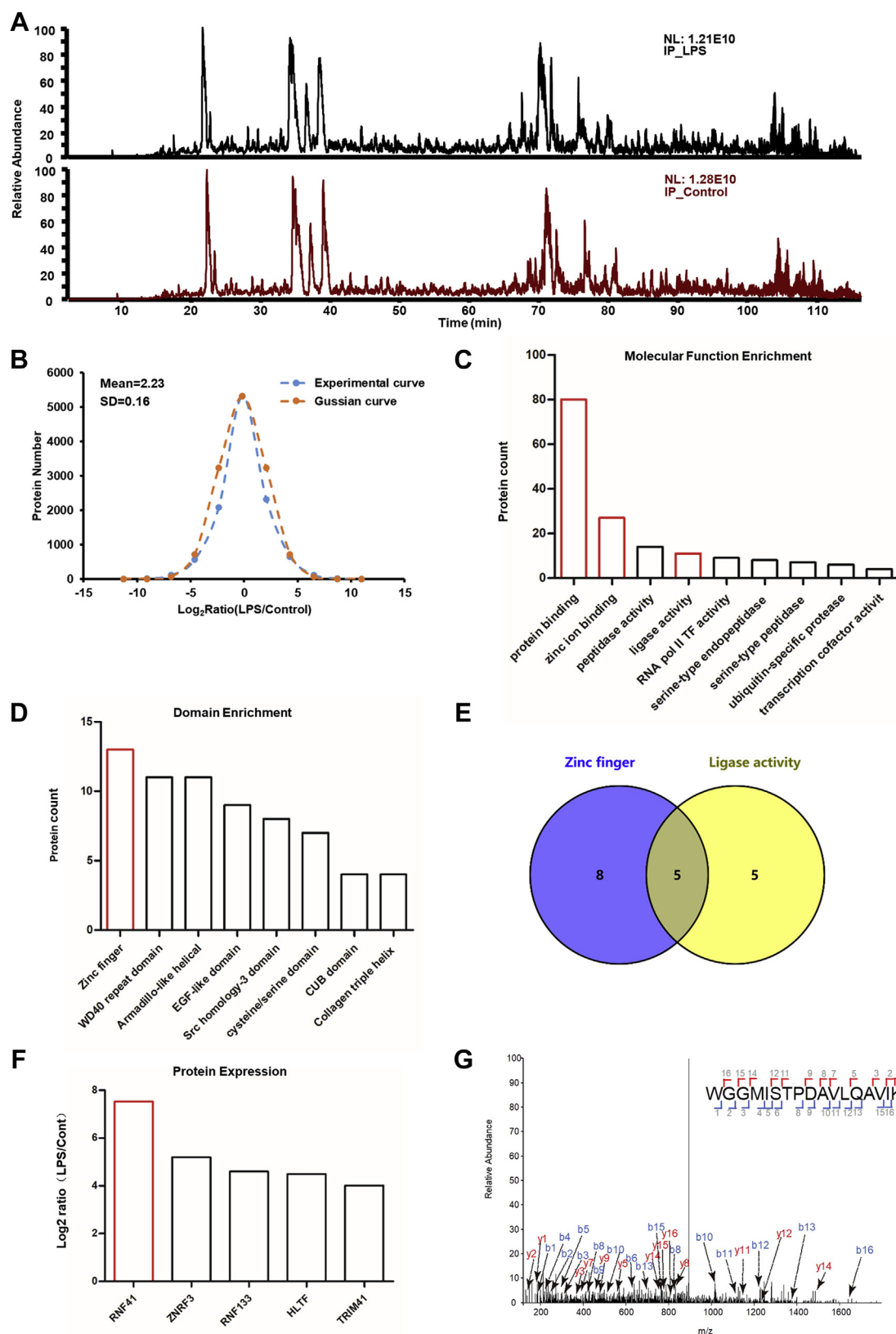


Figure 1. Screening for potential A20-binding proteins by co-IP/MS. *A*, base peak spectra of the LPS stimulation and control groups. *B*, histograms of log₂ ratios of quantified proteins in the experiment to screen differentially expressed proteins (DEPs) between the LPS stimulation and control groups. *C*, functional enrichment analysis of upregulated DEPs. *D*, analysis of the protein domain characteristics of upregulated DEPs. *E*, the expression levels of proteins with ubiquitin ligase activity and a zinc finger domain were compared to screen for potential A20-binding proteins. *F*, comparison of the expression of potential A20-interacting proteins. *G*, characteristic peptide spectrum of Nrdp1. NL, normalization level.

A20 regulates the polyubiquitination of Nrdp1

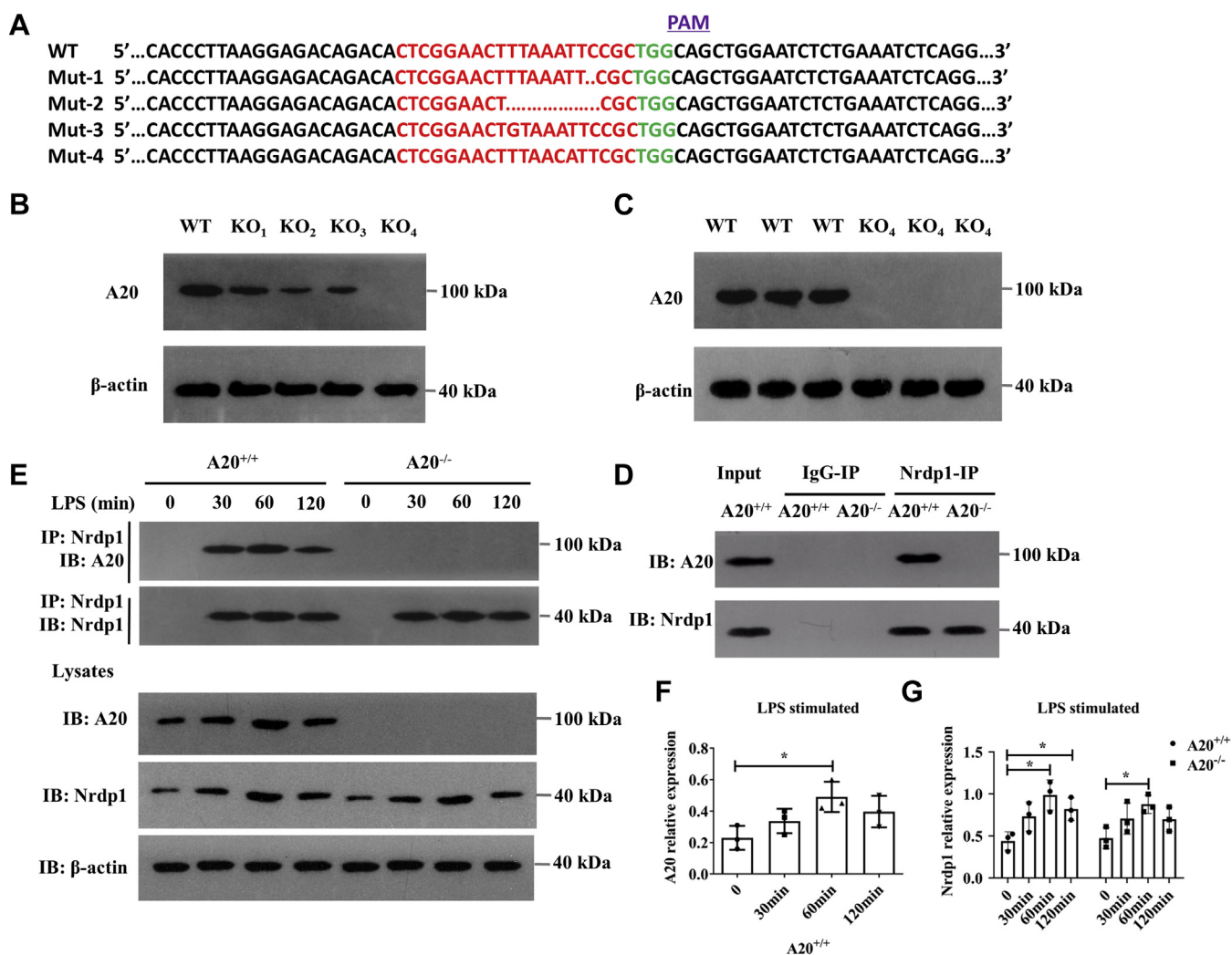


Figure 2. A20 interacted with Nrdp1 in macrophage cells. *A*, the A20 sequence was knocked out by CRISPR/Cas9 gene editing technology. *B*, silencing efficiency of four sgRNA sequences targeting the A20 gene was determined by western blot analysis. *C*, verification of the KO efficiency of the fourth sequence. *D*, specificity of the interaction of A20 and Nrdp1. Lysates were immunoprecipitated with an anti-Nrdp1 antibody or a control IgG antibody and detected by immunoblotting with antibodies against A20 and Nrdp1. *E*, A20^{+/+} and A20^{-/-} RAW264.7 cells were stimulated with LPS. After stimulation, the lysates were immunoprecipitated with an anti-Nrdp1 antibody and then immunoblotted with an anti-A20 antibody. Densitometry analysis was conducted following LPS stimulation for different durations. Relative A20 expression data were analyzed by one-way ANOVA, and relative Nrdp1 expression data were analyzed by two-way ANOVA. Values are the mean \pm SD of $n = 3$. * $p < 0.05$. NS, not significant.

signaling molecules MyD88 and TBK1 mediated by Nrdp1. The results showed that the expression of MyD88 and TBK1 in cell lysates was increased after A20 KO, especially when the cells were stimulated with LPS for 60 min (Fig. 3, *A* and *B*). To test whether Nrdp1 activation was affected by interaction with A20, we employed co-IP to examine any variations in the interactions between Nrdp1 and MyD88/TBK1. Importantly, A20 KO significantly inhibited the binding of Nrdp1 to MyD88 but increased the interaction of Nrdp1 with TBK1 (Fig. 3, *A* and *B*).

To examine antibody specificity, proteins from lysates were immunoprecipitated with an Nrdp1 antibody or a control rabbit antibody. As seen in Figure 3C, IP with the Nrdp1 antibody, but not the isotype control, yielded MyD88 and TBK1. Previous research has revealed that A20 restricts MyD88- and TBK1-independent signals through different molecules, such as TRAF6 and TRAF3 (24, 25), respectively.

We found that A20 can simultaneously inhibit MyD88 and TBK1 signals by binding Nrdp1, suggesting that Nrdp1 plays a key role in A20-mediated regulation of inflammatory signals.

A20 promotes Nrdp1-mediated K48-linked polyubiquitination to degrade MyD88

We verified the KO efficiency of Nrdp1 siRNA, and the results showed that the expression of Nrdp1 was significantly reduced at 72 h, indicating that it could be used in subsequent experiments (Fig. 4A). Then, we evaluated whether the changes in MyD88 expression were related to regulation of the ubiquitination process after A20 binds Nrdp1. After LPS stimulation, expression of the K48 ubiquitin chain of MyD88 was decreased in the A20-deficient groups, while MyD88 expression was correspondingly

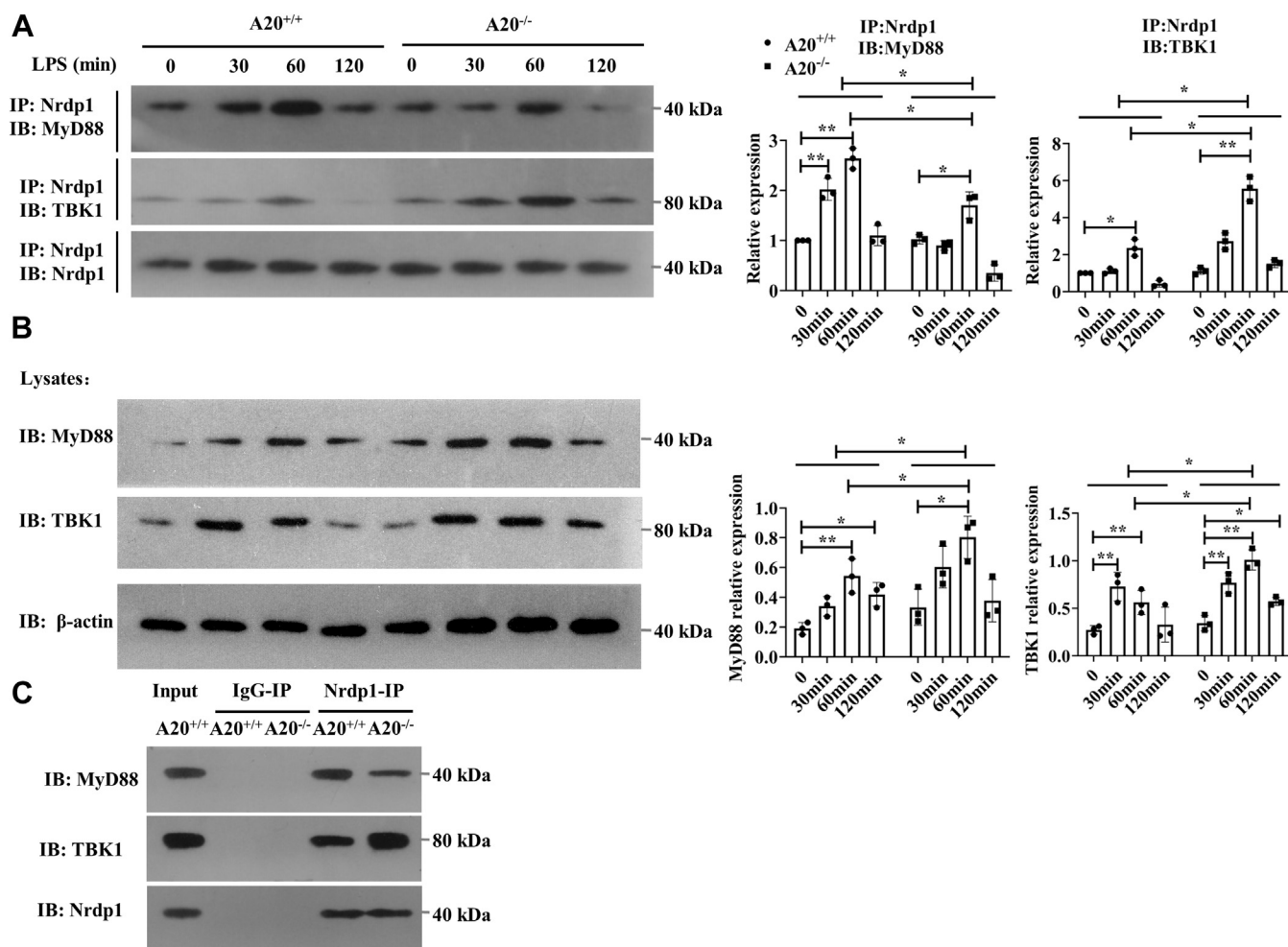


Figure 3. A20 participates in the Nrdp1-mediated inflammation signaling pathway. A, A20^{+/+} and A20^{-/-} RAW264.7 cells were stimulated with LPS, and proteins from the lysates were immunoprecipitated with an anti-Nrdp1 antibody and then immunoblotted with antibodies against MyD88 and Nrdp1. Densitometry data were analyzed with two-way ANOVA, and the values are the mean \pm SD of n = 3. *p < 0.05. **p < 0.01. B, the lysates were subjected to immunoblotting with antibodies against MyD88, TBK1, and GAPDH. Densitometry data were analyzed with two-way ANOVA, and the values are the mean \pm SD of n = 3. *p < 0.05. **p < 0.01. C, specificity of the interactions of A20 with MyD88, TBK1, and Nrdp1. A20^{+/+} and A20^{-/-} RAW264.7 cells were stimulated with LPS. Proteins from the lysates were immunoprecipitated with an anti-Nrdp1 or control IgG antibody and detected by immunoblotting with antibodies against MyD88, TBK1, and Nrdp1; n = 3.

increased, indicating that the K48 ubiquitin chain participates in the regulation of MyD88 expression and is regulated by A20. Importantly, when A20 and Nrdp1 expressions were suppressed at the same time, the signal for the K48 ubiquitin chain on MyD88 almost disappeared, indicating that the activity of the K48 ubiquitin chain on MyD88 is mediated by Nrdp1 (Fig. 4B).

Next, we investigated whether the activation of MyD88 after A20 KO would affect the activation of NF- κ B signaling. We selected three key molecules downstream of MyD88 in the NF- κ B signaling pathway (IRAK1, TAK1, and I κ B α) (26) and compared their expression. In the A20-KO group, due to the increased expression of MyD88, the expression of phosphorylated (p-) IRAK1, p-TAK1, and p-I κ B α was significantly increased (Fig. 4C). Consistent with this finding, when Nrdp1 was inhibited, there was no significant change in the expression of MyD88, resulting in little change in the expression of p-IRAK1, p-TAK1, and p-I κ B α compared with control levels (Fig. 4C). These results show that K48 ubiquitination of

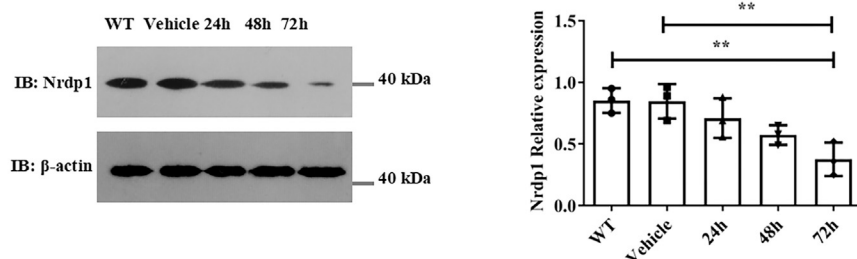
MyD88 was weakened after A20 KO, especially after Nrdp1 knockdown, suggesting that Nrdp1 plays an important role in the regulation of MyD88 ubiquitination by A20.

A20 inhibits the IFN pathway by reducing Nrdp1-mediated TBK1 activation

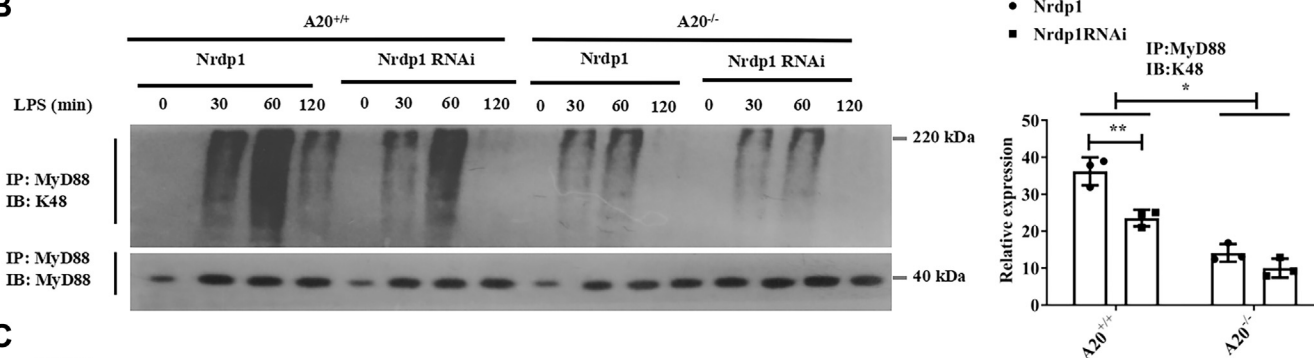
TBK1 is essential for IFN- β production and innate antiviral immunity. TBK1 activation is regulated in a variety of ways, including protein ubiquitination and phosphorylation. The E3 ubiquitin ligases MIBs, RNF128, and Nrdp1 have been found to activate TBK1 by promoting its K63-linked polyubiquitination (27, 28). In this study, the K63-linked ubiquitin chains of TBK1 were assessed to evaluate the effect of A20 on TBK1 activity through Nrdp1. In the A20-deficient groups, K63-linked ubiquitinated TBK1 accumulated, which led to the increased expression of TBK1 in lysates. In addition, the K63-linked polyubiquitination of TBK1 was reduced in both A20^{+/+} cells and A20^{-/-} RAW264.7 macrophages when Nrdp1 siRNA

A20 regulates the polyubiquitination of Nrdp1

A



B



C

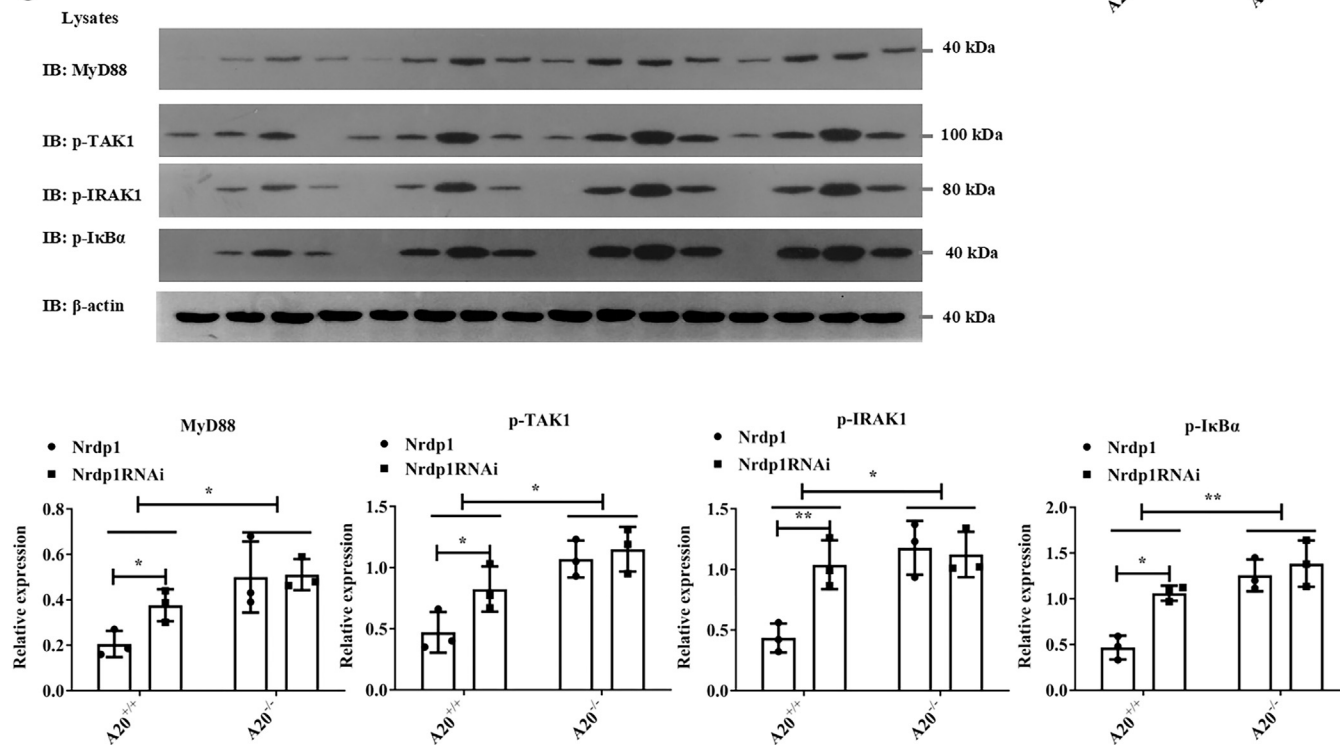


Figure 4. A20 promotes Nrdp1-mediated K48-linked polyubiquitination to degrade MyD88. *A*, comparison of Nrdp1 siRNA inhibition efficiency. Data were analyzed with one-way ANOVA, and values are the mean \pm SD of $n = 3$. $**p < 0.01$. *B*, $A20^{+/+}$ and $A20^{-/-}$ RAW264.7 cells were transfected for 72 h with control (scrambled) or Nrdp1 siRNAs. After transfection, the cells were treated with LPS for the indicated durations. Proteins from the lysates were immunoprecipitated with an antibody against MyD88 before being immunoblotted with a K48-specific antibody against ubiquitin and an antibody against MyD88. Densitometry data were analyzed with two-way ANOVA, and the values are the mean \pm SD of $n = 3$. $*p < 0.05$. $**p < 0.01$. *C*, the lysates were subjected to immunoblotting with antibodies against phosphorylated (p-) IRAK1, p-TAK1, p-IkBa, MyD88, and GAPDH. Densitometry data were analyzed with two-way ANOVA, and the values are the mean \pm SD of $n = 3$. $*p < 0.05$. $**p < 0.01$.

was stably transfected into the cells (Fig. 5A), indicating that Nrdp1 is a key molecule by which A20 regulates the activity of the K63 ubiquitin chain on TBK1.

Previous research has confirmed that activated TBK1 phosphorylates IRF3 (p-IRF3) and IRF7 to form a dimer that is

translocated into the nucleus to produce chemokines as well as small amounts of IFN- β (29). In this study, IRF3 phosphorylation was increased in the $A20^{-/-}$ group (Fig. 5B), further indicating that A20 negatively regulates the effects of Nrdp1 on TBK1 activation.

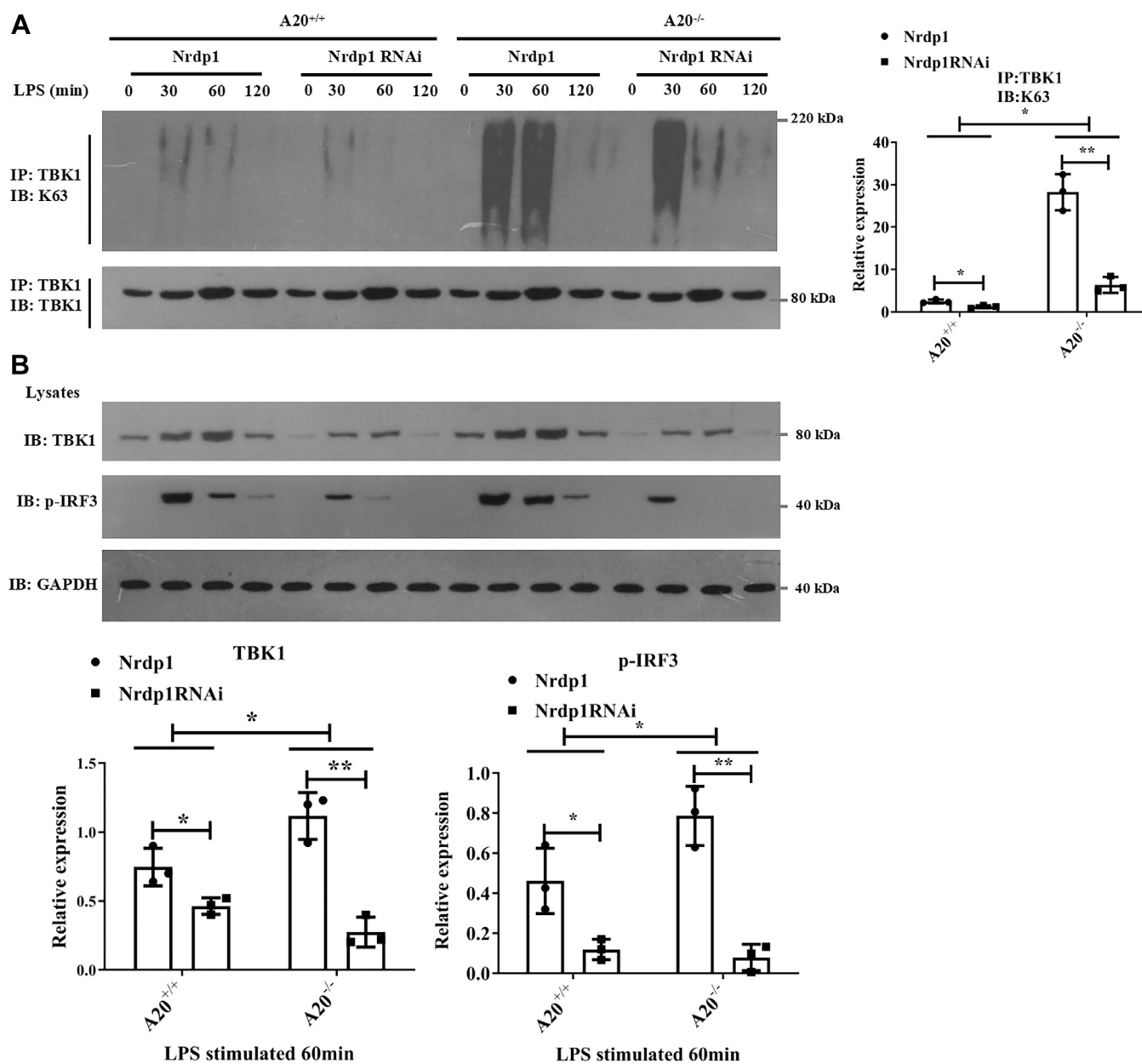


Figure 5. A20 inhibits the IFN pathway by reducing Nrdp1-mediated TBK1 activation. A, A20^{+/+} and A20^{-/-} RAW264.7 cells were transfected for 72 h with control (scrambled) or Nrdp1 siRNA. After transfection, the cells were treated with LPS for the indicated durations. Proteins from the lysates were immunoprecipitated with an antibody against TBK1 and then immunoblotted with a K63-specific antibody against ubiquitin (ubi) and an antibody against TBK1. B, the lysates were subjected to immunoblotting with antibodies against the K63-ubi blot panel, phosphorylated (p-) IRF3, TBK1, and GAPDH. Densitometry data were analyzed with two-way ANOVA, and the values are the mean \pm SD of n = 3. **p* < 0.05. ***p* < 0.01.

The ZnF4 domain of A20 is required for the binding of A20 and Nrdp1

Next, we examined the role of the A20 OTU (an N-terminal OTU DUB domain) and ZnF motifs in the interaction between A20 and Nrdp1 induced by LPS (Fig. 6A). Different OTU regions or ZnF motifs of A20 were transferred into A20-KO cells, and their effects on the ability of A20 to bind Nrdp1 were compared (Fig. 6B). The interaction failed to occur after transient transfection of Flag-A20 (1–367) into A20-KO cells, indicating that this OTU region does not affect the interaction between A20 and Nrdp1. Similarly, there was no interaction between A20 and Nrdp1 after transfection of Flag-A20

(1–590) into A20-KO cells, indicating that this OTU and ZnF1 to 3 do not affect the interaction between A20 and Nrdp1. However, an interaction was observed when Flag-A20 (547–775) was transfected, which indicates that the ZnF4 to 7 motifs play key roles in the interaction between A20 and Nrdp1 (Fig. 6B). Furthermore, there was no interaction between A20 and Nrdp1 when both ZnF4 and ZnF5 were deleted from wild-type cells (Δ 547–699, Fig. 6C) or when ZnF4 was deleted alone (Δ 590–640, Fig. 6C), indicating that ZnF4 plays a key role in the interaction between A20 and Nrdp1.

Since NF- κ B activation typically occurs *via* degradation of the NF- κ B inhibitory protein I κ B α (26), the I κ B α was assessed

A20 regulates the polyubiquitination of Nrdp1

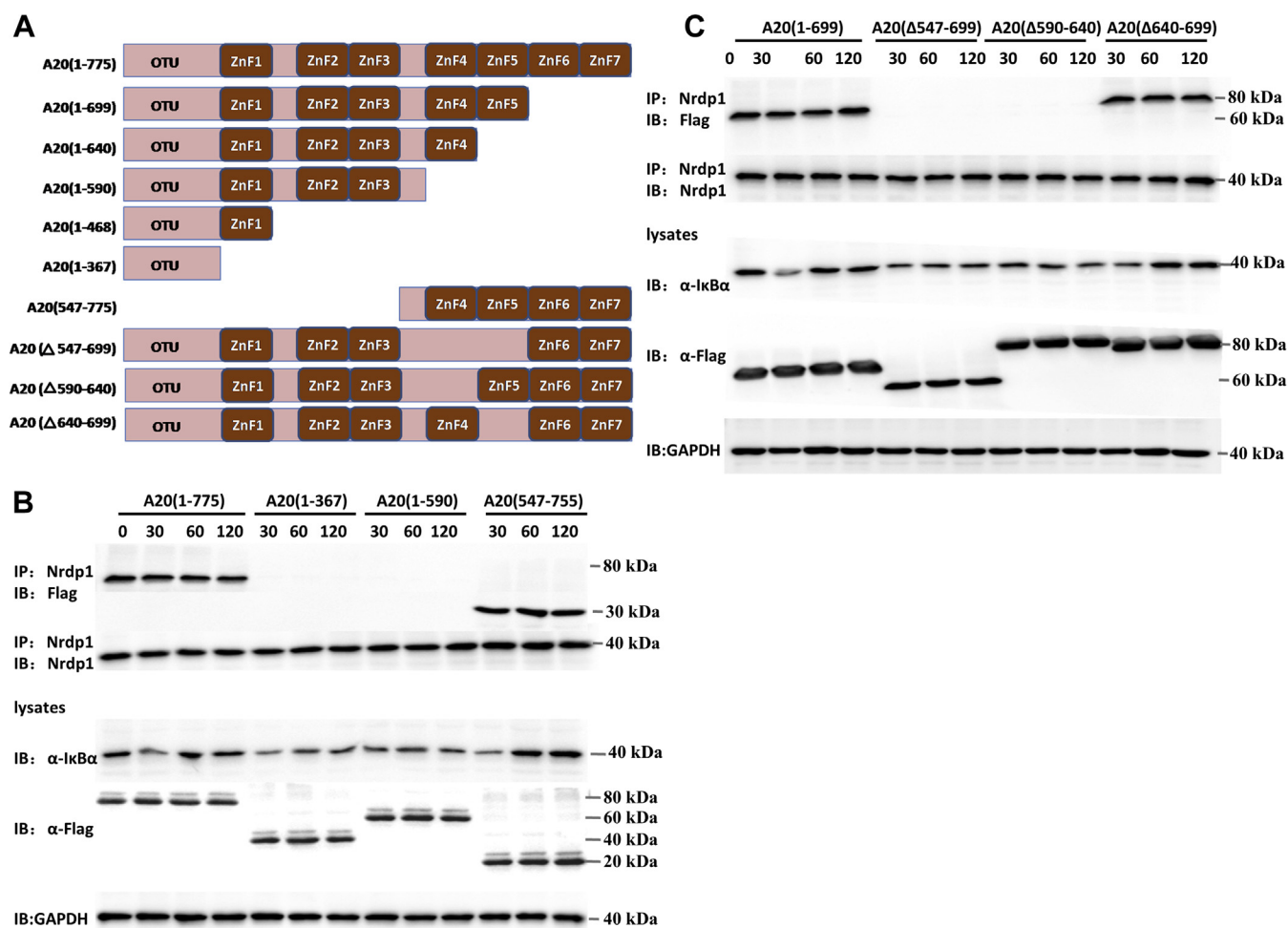


Figure 6. The ZnF4 domain of A20 is required for the binding of A20 and Nrdp1. A, schematic of A20 deletion mutants. B and C, A20^{-/-} RAW264.7 cells were transiently transfected with the indicated mutants. After 48 h, the cells were treated 60 min with LPS for the indicated durations. The lysates were subjected to immunoblotting with antibodies against Nrdp1, α-IκBα, GAPDH, and Flag.

to detect whether the binding of different fragments of A20 and Nrdp1 would also affect the activation of NF-κB signaling. Similar to previous results, the expression of IκBα was the highest when Flag-A20 (547–775) was transfected into the cells, and the expression level decreased after the deletion of ZnF4. These results reveal that the ZnF4 domain of A20 is a key region for Nrdp1 binding that regulates the downstream NF-κB signaling pathway.

Nrdp1 preferentially promotes the production of IFN-β

Since A20 KO leads to rapid death in animals, we evaluated the inflammatory effect of Nrdp1 in the context of A20 regulation at the cellular level. According to our previous research (30) and other studies (31–33), IL-6, TNF-α, and IFN-β can be used as indicators to assess the severity of inflammation. Under LPS stimulation, the expression of proinflammatory cytokines (IL-6, TNF-α, IFN-β) was significantly increased in the A20-KO group, confirming that A20 has a wide range of inhibitory effects on proinflammatory cytokines. However, the inhibition of Nrdp1 alone increased the secretion of IL-6 and TNF-α but decreased IFN-β secretion, suggesting

that Nrdp1 preferentially promotes the production of IFN-β (Fig. 7, A–C). In addition, the expression of IL-6 and TNF-α was not significantly changed, but IFN-β secretion was significantly decreased when compared with that in the A20^{-/-} and A20^{-/-} of Nrdp1 siRNA groups (Fig. 7, A–C). The above results indicated that A20 tends to inhibit the expression of IL-6 and TNFα, while the inhibition of IFN-β expression by A20 mainly depends on the polyubiquitination regulation of Nrdp1. Moreover, Nrdp1 may play a dominant role in regulating the expression of IFN-β when compared with A20.

Discussion

Previous studies have confirmed that A20 inhibits inflammatory response by negatively regulating TLRs signaling. TLRs-mediated signaling is divided into largely two pathways: MyD88-dependent and -independent pathways (TRIF-dependent pathway). The MyD88-dependent pathway sequentially uses the IRAK kinases, the ubiquitin ligase TRAF6, and the kinase TAK1 to early-phase activation of NF-κB and the transcription of genes encoding various proinflammatory cytokines including IL-6, TNF, and IL-1β. Moreover, the TRIF-

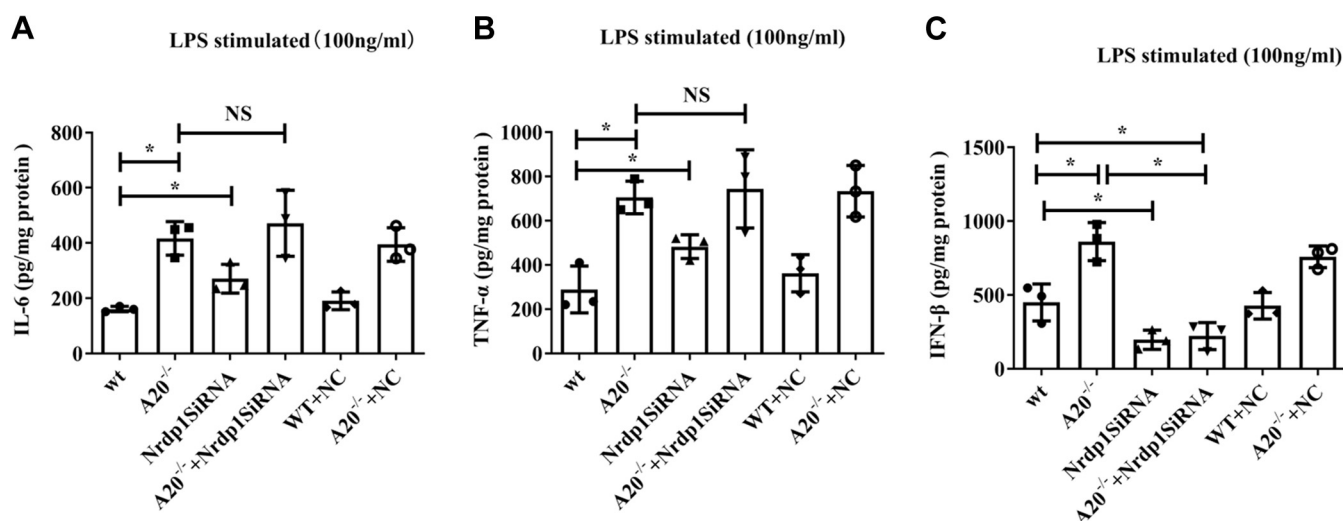


Figure 7. Nrdp1 regulates the A20-mediated inflammatory response. The expression of IL-6 (A), TNF-α (B), and IFN-β (C) induced by LPS stimulation for 60 min in A20^{+/+} and A20^{-/-} RAW264.7 cells was measured by ELISA. **p* < 0.05. NC, empty plasmid without the Nrdp1 sequence; NS, no significance; WT, wild-type (control group).

dependent pathway signals through a cascade of TRIF, the kinase TBK1, the adaptor TRAF3, and the transcription factor IRF3, resulting in the production of IFN-β (Fig. 8) (34, 35).

In this study, co-IP coupled with MS was used to profile A20-binding proteins to clarify the molecular mechanism of A20. Bioinformatics screening and filtering identified Nrdp1 as

a potential A20-interacting molecule. Subsequent experimental results confirmed that endogenous Nrdp1 interacts with A20 in a stimulus-dependent manner. These findings indicate that Nrdp1 is an essential regulator of A20 and therefore most likely a novel subunit of the A20 ubiquitin-editing complex.

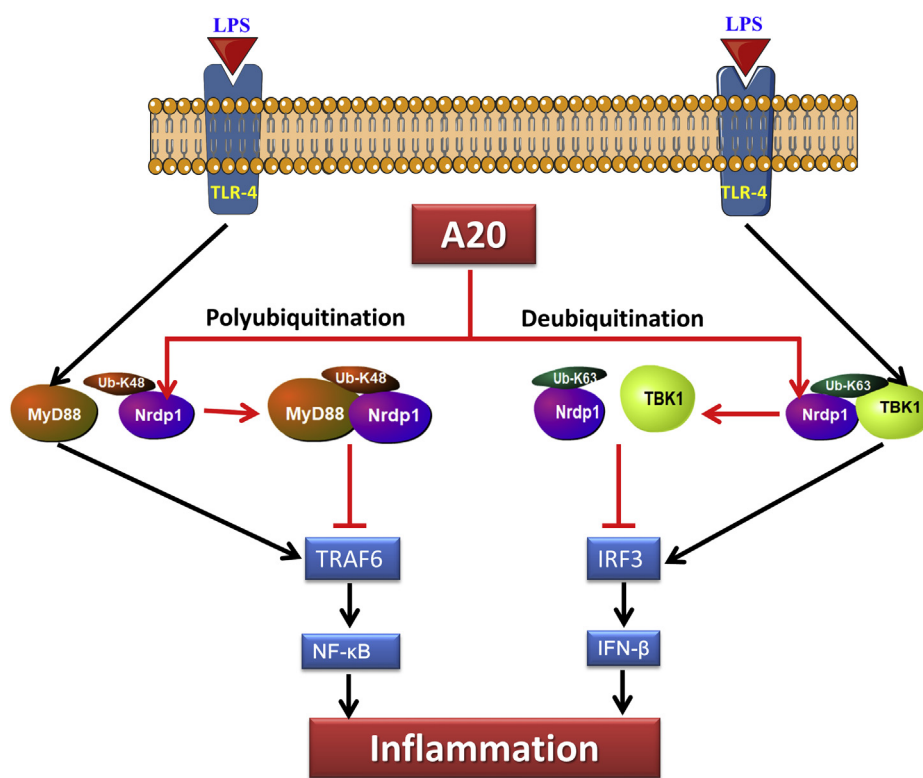


Figure 8. Schematic illustration showing A20 regulation on Nrdp1-dependent signaling pathways. The black arrow indicates the known inflammatory signaling pathway, while the red line illustrates the inflammatory signaling pathway regulated by Nrdp1 interaction with A20. Our study reveals that A20 inhibits inflammation through two ways. On one hand, A20 promotes the polyubiquitination of Nrdp1 and then induces the degradation of MyD88, inhibiting the activation of NF-κB through downregulation of TRAF6. On the other hand, A20 may also promote the deubiquitination of Nrdp1 and then deactivates TBK1, inhibiting the secretion of IFN-β through downregulation of IRF3.

A20 regulates the polyubiquitination of Nrdp1

Nrdp1 belongs to the family of single RING finger-containing proteins that function as E3 ubiquitin ligases. Nrdp1 serves as a scaffold by coordinating ubiquitin transfer from a ubiquitin-conjugating enzyme (E2) recruited by its N-terminal RING domain to a specific substrate that interacts with its C-terminal substrate-binding domain (36). Nrdp1 was also suggested to be involved in the ubiquitylation and degradation of two other E3 ubiquitin ligases: BRUCE (37), an antiapoptotic protein, and parkin (38), a protein involved in the onset of Parkinson's disease. Additionally, Nrdp1 has been found to negatively regulate MyD88-dependent activation of NF- κ B by catalyzing K48-linked ubiquitination of MyD88 (23). On this basis, we confirmed that Nrdp1-mediated K48-linked polyubiquitination of MyD88 for its degradation is promoted by the binding of Nrdp1 to A20. Upon LPS or IL-1 β stimulation, several adaptor proteins, including MyD88 and TRAF6, are recruited to the receptor. A20 inhibits NF- κ B signaling by removing K63-linked polyubiquitin chains from TRAF6 and disrupts the interaction of TRAF6 with the ubiquitin-conjugating enzymes Ubc13 and UbcH5c; K48-linked polyubiquitination and the degradation of Ubc13 and UbcH5c subsequently occur (39). However, the molecular mechanism by which the TRAF6 upstream molecule MyD88 is regulated by A20 has not yet been clarified. In this study, we found that A20 affects the activity of MyD88 by binding Nrdp1, which provides a new molecular mechanism by which A20 regulates the NF- κ B signaling pathway.

Moreover, IFN production is tightly regulated by a variety of negative regulators to maintain homeostasis and prevent autoimmunity. Previous studies have demonstrated that A20 together with TBK1 and IKKi inhibits IFN- β production (40, 41). Then, activated TBK1 phosphorylates IRF3 to form a dimer, and nuclear translocation occurs, resulting in type I IFN gene activation (25). However, the mechanism by which A20 affects IFN signaling by regulating TBK1 is unclear. In this report, we demonstrate that A20 inhibits the Nrdp1-mediated activation of TBK1 and then inhibits IFN- β secretion, revealing a new mechanism by which A20 inhibits inflammation and IFN- β secretion.

Furthermore, the ZnF4 domain has been shown to act as an ubiquitin-binding domain (UBD) for K63-linked polyubiquitin (42). Deletion of ZnF4 in A20 mice increased the K63-linked ubiquitination of RIPK1 and TNFR1 to activate NF- κ B signaling upon TNF stimulation (43). Mechanistically, it is probably that when A20 (547–775) is binding to Nrdp1, the Nrdp1 turns on the polyubiquitination function on the MyD88 to enhance its degradation. Secondly, we found that the complex of A20 (547–775) and Nrdp1 could enhance the degradation of MyD88 and then cause the elevation of I κ B α in the lysate. In our study, the ZnF4 domain of A20 was required for Nrdp1 polyubiquitination. These findings indicate that ZnF4 is an essential domain of A20 that allows it to bind Nrdp1.

The interacting protein dataset also revealed additional molecules potentially involved in the A20 inflammatory response process. As shown in Figure 1E, other proteins that both contain ZnF domains and exhibit ubiquitin ligase activity

may be potential A20-binding proteins. For example, ZNRF3 is an E3 ubiquitin-protein ligase that acts as a negative regulator of the Wnt signaling pathway by mediating the ubiquitination and subsequent degradation of the Wnt receptor complex components Frizzled and LRP6 (44). TRIM41 contains a RING finger domain and functions as an E3 ligase that catalyzes the ubiquitin-mediated degradation of protein kinase C (44). A recent study reported that TRIM41 modulates α -synuclein expression by regulating ZSCAN21 and that TRIM41 variants exist in patients with familial Parkinson's disease (45). Whether A20 can regulate its own signaling pathways and disease processes by binding these proteins requires further study.

However, this study has several limitations. First, A20 KO experiments were conducted only *in vitro* and not *in vivo* since A20-KO mice die prematurely (4, 5). Mice lacking A20 die shortly after birth due to massive multiorgan inflammation, which confirms the key role of A20 in immune homeostasis (4). Our previous research revealed that A20^{-/-} mice with intracerebral hemorrhage-induced inflammatory injury exhibited increased hematoma volumes and proinflammatory factor levels (30). We also noticed that only 40% of A20^{-/-} mice survived to 8 weeks of age, and the remaining mice died less than a week after intracerebral hemorrhage, similar to the situation in a previous study (5). In addition, we intraperitoneally injected LPS into A20-KO mice, but the mice died too quickly to continue the follow-up experiment. These data suggest that A20 KO likely causes severe inflammation or even death *in vivo*.

In summary, previous studies have found that Nrdp1 directly bound and polyubiquitinated MyD88 and TBK1, which led to degradation of MyD88 and activation of TBK1 (23). This study confirms the role of A20 in Nrdp1-mediated regulation of ubiquitination. A20 can promote the interaction between Nrdp1 and MyD88 and lead to the degradation of MyD88, thus inhibiting the activation of NF- κ B. In addition, A20 inhibits Nrdp1-mediated TBK1 activation and subsequent IFN- β secretion (Fig. 8). Our experiments reveal a new mechanism by which A20 inhibits inflammation and IFN- β secretion.

Experimental procedures

Cell culture and stimulation

The mouse macrophage cell line RAW264.7, obtained from the Cell Bank of the Chinese Academy of Sciences, was cultured in DMEM supplemented with 10% fetal bovine serum and a glutamine–penicillin–streptomycin mixture (all from Gibco, Life Technologies) in a 37 °C humidified 5% CO₂ incubator. For LPS (100 ng/ml, Sigma) stimulation, 2 × 10⁶ cells were cultured for 0, 30, 60, or 120 min at 37 °C in culture medium.

Generation of A20-KO cells by CRISPR/Cas9

Four single-guide RNAs (sgRNAs) that specifically recognize the A20 gene were designed and synthesized. The sgRNAs were cloned into the pX458 vector containing an ampicillin

selection cassette and GFP (Addgene) according to the manufacturer's guidelines (46). Then, 2 μ l of the ligated plasmid was transformed into competent cells, which were then plated for selection, and single colonies were expanded with plasmid extraction using a Maxiprep kit (Qiagen). Colonies for which the sgRNA sequence had been inserted correctly were confirmed by Sanger sequencing. Then, 1×10^6 cells were transfected with 10 μ g of pX458 vector containing sgRNA together with transfection reagent (Invitrogen). Forty-eight hours after transfection, GFP-positive cells were single-cell sorted into 96-well plates. The cells were left to proliferate and screened for the presence or absence of the A20 protein by western blot analysis.

Protein extraction and co-IP

LPS (100 ng/ml) was added to A20^{+/+} and A20^{-/-} RAW264.7 cells when they reached approximately 90 to 100% confluency. The cells were collected and washed three times with cold PBS at different time points according to the experimental design. Then, the cells were suspended in 500 μ l of cold PBS (pH 7.2) and disrupted with a Soniprep sonicator (Scientz) at 25% power for eight cycles. The cell lysates were centrifuged at 17,000g for 20 min. The extracted protein solutions were quantified with a BCA protein assay kit (Pierce) so that lysates containing equal amounts of protein could be used for all subsequent experiments.

Co-IP was performed as described previously (47). Two microliters of a prepared A20 antibody solution (1:100) was added to beads (Thermo Fisher Scientific), which were incubated for 30 to 60 min at room temperature. The beads were collected to remove the supernatant and washed twice. Then, the protein lysates were added to the bead mixtures. The beads and lysates were gently mixed and incubated on a rotating platform for 2 h at 4 °C. Next, the beads with bound antibodies were collected with a magnetic stand. The above operation was repeated twice to fully enrich the immunoprecipitated sample. Next, 500 μ l of IP lysis/wash buffer (Thermo Fisher Scientific) was added, and the beads were gently vortexed and collected to obtain the immunoprecipitates. Finally, the products containing A20-interacting proteins were obtained by centrifugation at 12,000g for 5 min at 4 °C. The precipitates were subjected to SDS-PAGE and MS analysis as described below.

Identification of A20-interacting proteins by MS

Co-IP with MS was performed as described previously (48). The protein concentration of each total-cell lysate (TCL) was measured by Coomassie staining of SDS-PAGE gels. Proteins in a certain amount of TCL were separated through SDS-PAGE, and the gel was cut into approximately 1 mm³ pieces for decolorization and dehydration and then digested with 10 ng/ μ l trypsin (Promega) for 16 h at 37 °C. Then, the digested peptides were extracted in extraction buffer (5% formic acid and 45% acetonitrile) and completely dried with a vacuum dryer (Labco, CENTRIVAP).

All samples were analyzed by an online nanospray Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) coupled with an EASY-nLC system (Thermo Fisher Scientific). The peptides were redissolved in solvent A (0.1% formic acid in water). One microgram peptide sample was loaded to an Acclaim PepMap C18 column (75 μ m \times 25 cm) and separated with a 120-min linear gradient. The column flow rate was maintained at 600 nl/min and the column temperature was maintained at 40 °C. The electrospray voltage of 2100 V was used. The full scan was performed for m/z 350 to 1200 with the resolution of 120,000 at m/z = 200, and the maximum injection time was 50 ms. The MS/MS scan was performed with higher-energy collision-activated dissociation for m/z 200 to 2000 with the resolution of 30,000 at m/z = 200, and the maximum injection time was 35 ms. The collision energy was 32%, and the stepped collision energy was 5%.

Data-dependent acquisition (DDA) was analyzed using Proteome Discoverer 2.1.0182 (Thermo Fisher Scientific). MS1 tolerance was set to 10 ppm, and MS/MS tolerance was 0.02 Da. All the DDA MS/MS spectra were searched against the database of mouse proteomes downloaded from <https://www.uniprot.org/> (84,646 protein entries, access date July 2020). Trypsin was the digestion enzyme. Carbamidomethyl (C) was considered as fixed modification. Oxidation (M), Acetyl (Protein N-term), and Deamidated (N, Q) were considered as variable modifications. A peptide-spectra match (PSM) was performed on a mini peptide length of seven amino acids; precursor ion spectra were set at a tolerance of 20 ppm; peaks with a signal-to-noise value below the threshold 1.5 will be removed; the false discovery rate (FDR) allowing for credible proteins and peptides was less than 1.0% and two missed cleavages were permitted.

The Proteome Discoverer (version 2.1.0182) compares the raw data taken from mass to the information from a selected FASTA database and identifies proteins from the mass spectra of digested fragments. Specifically, the application does the following: Works with peak-finding search engines such as Sequest HT to process the raw MS data and generates a peak list and relative abundances. The peaks represent the fragments of peptides for a given mass and charge. Complementary data was produced from a variety of dissociation methods and data-dependent stages of tandem MS. The search engines correlate the uninterrupted tandem mass spectra of peptides with FASTA database. Protein abundance is calculated by summing sample abundance of the peptide groups.

The proteins were quantified on the basis of the reporter ion intensity of the identified unique peptides. Proteins with unique peptide numbers greater than 2 were included in quantitative analysis. By fitting all datasets to a Gaussian distribution, we regard the scattered proteins as differentially expressed proteins according to the previously reported proteomic threshold method (21, 49). Pathway enrichment analysis of the DEPs was performed through a combination of GO analysis, Database for Annotation, Visualization and Integrated Discovery (DAVID) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (50). The MS/MS spectra were extracted and analyzed with the

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Xcalibur Qual Browser (v2.2) (Thermo Fisher Scientific) and pFind (v3.0) (51).

Cloning and expression of A20 fragment peptides

Then, the A20 peptide-encoding DNA fragments A20 (1–775), A20 (1–367), A20 (1–590), A20 (547–775), A20 (1–699), A20 (Δ 547–699), A20 (Δ 590–640), and A20 (Δ 640–699) were synthesized and cloned into the pCMV-Flag vector (Beyotime) *via* the BmaHI and XbaI sites. A20 knockout RAW264.7 cells were cotransfected with 500 ng of the constructed plasmid by using Lipofectamine 2000 transfection reagent as recommended by the manufacturer. Purification of the recombinant peptides was performed according to a previously described procedure (42).

Western blot analysis and detection of polyubiquitin linkages

In each experiment, lysates containing equal amounts of protein were separated by SDS-PAGE, and the separated proteins were transferred onto polyvinylidene fluoride membranes by electroblotting (Bio-Rad). Western blotting was carried out based on our previously reported method (52). The antibodies used in this study are shown in Table 1.

RNA interference

Small interfering RNA (siRNA) was designed and used to silence Nrdp1 as described previously (47). A20^{+/+} and A20^{-/-} RAW264.7 cells were transfected with different Nrdp1 siRNAs at 50 nM or nonsilencing siRNA (GeneChem Co Ltd) as a negative control using Lipofectamine 2000 transfection reagent (Invitrogen) as recommended by the manufacturer.

ELISA

ELISA was performed as described previously (52). The supernatants from LPS-stimulated RAW264.7 cells were assessed by ELISA according to the manufacturer's instructions (R&D). A standard curve was established as a reference basis for the concentration of the substance to be tested in the samples.

Statistical analyses

Statistical analyses were performed using Prism7 software. Data are presented as the mean \pm standard deviation (SD) from three independent experiments. One-way analysis of

variance followed by Tukey's post hoc test was used to compare the expression of A20 or Nrdp1 after LPS treatment for different durations. Two-way analysis of variance followed by the Sidak post hoc test was used to compare WT or A20^{-/-} macrophages treated with Nrdp1 siRNA. A *p*-value of less than 0.05 indicated significance.

Data availability

The MS proteomics data has been uploaded to iProX (<https://www.iprox.org>) with the dataset identifiers IPX0002821000.

Supporting information—This article contains [supporting information](#).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ABIN1, A20-binding inhibitor of NF- κ B; co-IP, coimmunoprecipitation; DEP, differentially expressed protein; DUB, deubiquitination; GO, gene ontology; IFN, interferon; KO, knockout; LPS, lipopolysaccharide; MS, mass spectrometry; Nrdp1, neuregulin receptor degradation protein-1; OTU, ovarian tumor; p, phosphorylated; RIPK1, receptor-interacting serine/threonine protein kinase 1; RNF41, ring finger protein 41; TAX1BP1, Tax1-binding protein 1; TPK1, threonine protein kinase; TLR4, Toll-like receptor 4; TNFR, tumor necrosis factor receptor; TRAF6, TNFR-associated factor 6; ZnF, zinc finger.

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Table 1
Antibodies used for western blot analysis

Antibody	Catalog no.	Dilution	Company
A20	166692	1:500	Santa Cruz
Nrdp1	374120	1:500	Santa Cruz
p-TAK1	09404	1:500	Abcam
β -actin	6276	1:2000	Abcam
MyD88	AF3109	1:500	R&D Systems
TBK1	3504	1:500	CST
K63-Ubi	12930	1:1000	CST
K48-Ubi	8081	1:1000	CST
I κ B α	4814	1:500	CST
p-I κ B α	8219	1:500	CST
p-IRAK1	218130	1:500	Abcam

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