## Immunity

## A20 Restricts Ubiquitination of Pro-Interleukin-1 $\beta$ **Protein Complexes and Suppresses NLRP3 Inflammasome Activity**

#### **Highlights**

- A20-deficient macrophages exhibit NLRP3 inflammasome activity in response to LPS
- IL-1β co-IPs with RIPK1, RIPK3, caspase-1, and caspase-8 in ubiquitinated complexes
- IL-1β-associated ubiquitination is increased in A20-deficient cells and requires RIPK3
- K133 of IL-1β is a physiological ubiquitination site that supports IL-1β processing

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#### In Brief

The NF-κB inhibitor A20 prevents human inflammatory disease and lymphomas. Ma and colleagues show that A20 restricts pro-IL-1 ß ubiquitination to limit spontaneous NLRP3 inflammasome activation.





# A20 Restricts Ubiquitination of Pro-Interleukin-1 $\beta$ Protein Complexes and Suppresses NLRP3 Inflammasome Activity

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#### SUMMARY

Inappropriate inflammasome activation contributes to multiple human diseases, but the mechanisms by which inflammasomes are suppressed are poorly understood. The NF-kB inhibitor A20 is a ubiquitinmodifying enzyme that might be critical in preventing human inflammatory diseases. Here, we report that A20-deficient macrophages, unlike normal cells, exhibit spontaneous NLRP3 inflammasome activity to LPS alone. The kinase RIPK3, but not the adaptor MyD88, is required for this response. In normal cells, A20 constitutively associates with caspase-1 and pro-IL-1 $\beta$ , and NLRP3 activation further promotes A20 recruitment to the inflammasome. Pro-IL-1ß also co-immunoprecipitates with RIPK1, RIPK3, caspase-1, and caspase-8 in a complex that is modified with K63-linked and unanchored polyubiquitin. In A20-deficient macrophages, this pro-IL-1β-associated ubiquitination is markedly increased in a RIPK3-dependent manner. Mass spectrometric and mutational analyses reveal that K133 of pro-IL-1 $\beta$  is a physiological ubiquitination site that supports processing. Our study reveals a mechanism by which A20 prevents inflammatory diseases.

#### INTRODUCTION

Secretion of the pro-inflammatory cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 is normally regulated by at least two distinct signals (Martinon et al., 2002). First, expression of the inactive pro-proteins requires nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity transduced by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs). Subsequently, the proteolytic processing of these cytokines usually requires caspase-1 (Casp1) activation by cytosolic PRRs possessing a pyrin domain and/or caspase activation and recruitment domain (CARD) (Davis et al., 2011; Martinon et al., 2009). Upon detecting the presence of microbes or "danger signals," these proteins recruit Casp1 either directly or via the adaptor protein, ASC (apoptosis-associated speck-like protein containing a C-terminal CARD), to form a signaling



complex called the inflammasome. Though critical for efficient pathogen clearance, exaggerated inflammasome functions can play pathogenic roles in various autoimmune, allergic, and inflammatory disorders (Lamkanfi and Dixit, 2012; Strowig et al., 2012). The mechanisms by which inflammasomes are regulated are incompletely understood.

The NLRP3 (nucleotide-binding domain and leucine-rich repeats containing pyrin domain 3) inflammasome is normally induced by large particulates such as uric acid crystals, cholesterol crystals, amyloid or misfolded protein, and asbestos (Leemans et al., 2011). Exaggerated activities of NLRP3 to these particulates are implicated in the pathogenesis of gout, type-2 diabetes, atherosclerosis, neurodegenerative diseases, and asthma (Davis et al., 2011). How NLRP3 responds to such diverse stimuli is incompletely understood but might involve lysosomal rupture, reactive oxygen species (ROS), or mitochondrial damage (Leemans et al., 2011). The loss of intracellular K<sup>+</sup> ions appears to be required, as blocking K<sup>+</sup> efflux suppresses NLRP3 activities (Muñoz-Planillo et al., 2013). NLRP3 can also sense cellular damage from within or nearby cells by the release of ATP, which causes K<sup>+</sup> efflux through the P2X7 channel (Mariathasan et al., 2006). In response to Gram-negative bacteria, NLRP3 activation also requires the upregulation of Casp11 by type I interferons (IFNs) (Kayagaki et al., 2011; Rathinam et al., 2012). Although several molecules required for inflammasome activation have been described, inhibitors of these protein complexes have not been completely defined.

Covalent conjugation of ubiquitin (Ub) molecules to target proteins, termed ubiquitination, regulates protein stability and protein-protein interactions in signaling complexes. Poly-Ub chains can be linked via N-terminal amino groups on Met1 (termed linear chains) or via  $\varepsilon$ -amino groups on any of the seven lysines of Ub. These distinct poly-Ub conformations are recognized by specific Ub sensor proteins that facilitate the localization, activity, and interacting partners of ubiquitinated proteins (Corn and Vucic, 2014). In particular, K48-linked poly-Ub chains generally target proteins for proteasomal degradation, whereas linear and K63-linked chains primarily serve as a scaffolding network for the formation of signaling complexes.

A20, encoded by the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced protein 3 (*TNFAIP3*) gene, is a potent regulator of Ub-dependent signaling (Shembade and Harhaj, 2012; Verstrepen et al., 2010). Polymorphisms in the human *TNFAIP3* gene locus are

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## Figure 1. A20 Deficiency Causes Secretion of Mature IL-1 $\beta$ in the Absence of an Exogenous Second Signal for Inflammasome Activation

(A–C) BMDMs cultured from WT or *Tnfaip3<sup>-/-</sup>* mice were stimulated with the following ligands for 15 hr: 10 µg/mL Zym, 100 µg/mL poly(I:C) (P(I:C)), 500 ng/mL LPS, 3 µg/mL Gdq, and 5 µM CpG. (A) Supernatants were analyzed by ELISA for IL-1 $\beta$  secretion. (B) Whole-cell lysates were analyzed by IB for the indicated proteins after 6 hr stimulation of each ligand as in (A). (C) IL-1 $\beta$  was IP'd from supernatants and analyzed by IB to confirm proteolytic processing.

(D) IL-1 $\beta$  secretions by *Tnfaip3<sup>-/-</sup>* BMDMs in response to 500 ng/mL LPS for 0–18 hr, or 0–500 ng/mL LPS for 18 hr, were quantitated by ELISA.

(E) IL-1 $\beta$  secretions in response to Dectin-1, TLR3, and TLR4 ligands by A20-sufficient and -deficient BMDMs on *Myd88<sup>-/-</sup>* background were measured after ~15 hr. Note TLR2 cannot respond to Zym without MyD88.

(F) IL-1 $\beta$  secretions by A20-deficient cells after  ${\sim}15$  hr of TNF- $\alpha$  or anti-CD40 stimulation were measured.

(G) Supernatant from A20-deficient cells stimulated with LPS for ~15 hr (bottom of graph) was transferred to LPS-primed control cells. After overnight incubation, the supernatant was measured again for any additional IL-1ß secretion by control cells. As controls for NLRP3 responsiveness, IL-1ß responses to ATP by control cells in the presence or absence K<sup>+</sup> efflux were measured. To eliminate any potential complications by autocrine TNF-a signaling, both control and A20-deficient cells used here were on Tnf-/ background. Each bar in graphs represents mean of triplicate wells + SD. \* denotes statistical significance by Student's t test (p < 0.05) when comparison is made between control and A20deficient cells for each condition. n.s. = not statistically significant. All data presented here and hereafter are representative of at least three independent experiments.

associated with a number of inflammatory diseases, suggesting that A20 prevents the incidence and/or severity of these diseases (Catrysse et al., 2014; Ma and Malynn, 2012). Correspondingly, A20-deficient mice spontaneously develop a systemic inflammatory syndrome that culminates in premature death (Lee et al., 2000), while cell-specific deletions of A20 cause diseases in mice reminiscent of those in humans (Chu et al., 2011; Hammer et al., 2011; Heger et al., 2014; Kool et al., 2011; Matmati et al., 2011; Tavares et al., 2010; Vereecke et al., 2010). Deficiency in MyD88 (myeloid differentiation primary response 88) or commensal depletion abrogates the severity of several of these conditions, indicating that A20 restricts TLR and IL-1-receptor (IL-1R) family signaling (Boone et al., 2004; Turer et al., 2008). Hence, A20 appears critically positioned at the interface of host responses to commensal microbes. Given the genetic links between inflammasomes and A20 with human inflammatory diseases, we have investigated the potential role of A20 in regulating inflammasomes.

#### RESULTS

#### A20 Prevents Spontaneous Secretion of IL-1 $\beta$ and IL-18

To determine whether A20 regulates inflammasome activity, we stimulated bone marrow-derived macrophages (BMDMs) from  $Tnfaip3^{-/-}$  and control mice with various TLR ligands. Consistent with the two-signal requirement for IL-1 $\beta$  secretion (Martinon et al., 2002), TLR ligands alone induced only the intracellular expression of pro-IL-1 $\beta$  in wild-type (WT) cells (Figures 1A and 1B). No mature IL-1 $\beta$  was detected in the supernatants (Figure 1A). Surprisingly, even without a second signal for inflammasome activation,  $Tnfaip3^{-/-}$  BMDMs secreted IL-1 $\beta$  in response to zymosan (Zym), poly(I:C), and LPS (Figure 1A). Similar results were obtained for the secretion of IL-18 (data not shown). Other TLR ligands induced the expression of pro-IL-1 $\beta$  in both  $Tnfaip3^{-/-}$  and WT cells but failed to induce its processing and secretion (Figures 1B and 1A). To determine whether the secreted IL-1 $\beta$  was proteolytically

mature, we immunoprecipitated (IP'd) IL-1 $\beta$  from the supernatants of TLR-stimulated *Tnfaip3<sup>-/-</sup>* cells and assessed the molecular weight of the secreted IL-1 $\beta$  by immunoblotting (IB). These experiments confirmed the IL-1 $\beta$  secretion in each case to be the processed 17 kDa form (Figure 1C). The secretion of IL-1 $\beta$  from *Tnfaip3<sup>-/-</sup>* cells was detected as early as 2 hr after LPS stimulation and at LPS doses as low as 5 ng/mL (Figure 1D). Thus, A20 prevents spontaneous pro-IL-1 $\beta$  processing and secretion in cells stimulated with Zym, poly(I:C), and LPS.

Zymosan can activate MyD88-dependent as well as-independent pathways through TLR2 and Dectin-1, respectively (Brown et al., 2002; Underhill et al., 1999), and both MyD88 and TRIF (TLR/IL-1R domain-containing adaptor inducing IFN) are involved in TLR4 responses to LPS (Hoebe et al., 2003). We therefore assessed the contribution of MvD88 to the spontaneous IL-1 $\beta$  secretion induced by these ligands in *Tnfaip*3<sup>-/-</sup> cells. Tnfaip3<sup>-/-</sup>Myd88<sup>-/-</sup> cells retained the ability to process and secrete IL-1ß upon Zym or LPS stimulation (Figure 1E). Predictably, the IL-1 $\beta$  responses of A20-deficient cells to poly(I:C) were unaffected by MyD88 deficiency, because TLR3 uses only TRIF to signal (Figure 1E). Hence, TRIF-dependent signals and other MyD88-independent signals are sufficient to drive pro-IL-1ß processing in the absence of A20. To determine whether other MyD88-independent signals beside TLR3 and Dectin-1 could induce IL-1 $\beta$  secretion, we stimulated *Tnfaip*3<sup>-/-</sup> cells with TNF-a or agonistic anti-CD40 antibody. We found that anti-CD40, but not TNF- $\alpha$ , triggered IL-1 $\beta$  secretion in these cells (Figure 1F). Additionally, TNF- $\alpha$  deficiency had no effects on IL-1ß secretion by A20-deficient cells in response to all of these stimuli (data not shown), ruling out involvement of TNF-a autocrine signaling.

TLR3 and TLR4 can trigger programmed necrosis under certain conditions (Feoktistova et al., 2011; He et al., 2011; Kaiser et al., 2013). Thus, secretion of IL-1 $\beta$  by *Tnfaip*3<sup>-/-</sup> cells could result from the release of danger-associated molecular patterns (DAMPs) by dying cells (e.g., ATP) to activate inflammasomes. To test whether  $Tnfaip3^{-/-}$  cells might release such ligands, we stimulated LPS-primed control BMDMs with supernatants from LPS-stimulated Tnfaip3<sup>-/-</sup> cells and assayed for additional IL-1ß production by control cells. We found that *Tnfaip3<sup>-/-</sup>* BMDM-derived supernatant failed to stimulate additional IL-1 $\beta$  secretion from *Tnfaip*3<sup>+/+</sup> cells (Figure 1G). In contrast, addition of ATP to these cells induced robust IL-1ß secretion that could be inhibited by preventing K<sup>+</sup> efflux, as predicted for NLRP3 activation (Figure 1G). Taken together, these experiments suggest that A20 suppresses spontaneous IL-1β production in a cell-autonomous fashion.

#### A20 Suppresses NLRP3 Inflammasome Functions

We next determined whether A20 could also inhibit NLRP3 responses to a second signal. Addition of ATP to A20-deficient cells after 4 hr of LPS stimulation induced massive IL-1 $\beta$  secretion compared to WT cells (Figure 2A). Hence, A20 limits the magnitude of NLRP3 inflammasomes triggered by LPS plus ATP, as well as preventing spontaneous IL-1 $\beta$  secretion. Besides cleaving pro-IL-1 $\beta$ /18, overt Casp1 activity can also cause pyroptosis, an inflammatory form of lytic cell death (Davis et al., 2011). Accordingly, considerably more *Tnfaip3*<sup>-/-</sup> cells

died in response to LPS plus ATP than control cells (Figure 2B). These results indicate that A20 also restricts pyroptosis induced by NLRP3 inflammasome activation. Of note, the amounts of cell death did not differ between WT and  $Tnfaip3^{-/-}$  cultures in response to LPS alone (Figure 2B), confirming that  $Tnfaip3^{-/-}$  cells did not die necrotically and release DAMPs as second signals for inflammasome activation (Figure 1G).

NLRP3, ASC, and Casp1 are all critical components of inflammasomes. To assess the potential contributions of each of these proteins to the spontaneous IL-1 $\beta$  secretion observed in A20-deficient cells, we interbred *Tnfaip3<sup>-/-</sup>* mice with *Nlrp3<sup>-/-</sup>*, *Asc<sup>-/-</sup>*, and *Casp1/11<sup>-/-</sup>* mice. BMDMs from all three compound mutant strains secreted 65%–100% less IL-1 $\beta$  than *Tnfaip3<sup>-/-</sup>* cells after stimulation with poly(I:C), LPS, or Zym (Figures 2C–2E). These results indicate that A20 restricts the spontaneous assembly of NLRP3 with ASC and Casp1 in response to these stimuli. Because Casp1-deficient mice also harbor mutant Casp11 allele from 129 strain (Kayagaki et al., 2011), the residual mature IL-1 $\beta$  secretions by *Tnfaip3<sup>-/-</sup> Casp1/11<sup>-/-</sup>* compound mutant cells indicate that A20-deficient cells can additionally process pro-IL-1 $\beta$  through a mechanism that is independent of both Casp1 and Casp11 (Figures 2C and 2E; data not shown).

Depletion of all three baculoviral inhibitor of apoptosis protein (IAP) repeat-containing enzymes (BIRC2, BIRC3, and BIRC4) in BMDMs has been reported to result in spontaneous NLRP3 inflammasome activation by a RIPK3 (receptor-interacting protein kinase 3)-dependent mechanism (Vince et al., 2012). We thus asked whether a similar pathway was responsible for the spontaneous NLRP3 activation in A20-deficient cells. We interbred *Tnfaip3<sup>-/-</sup>* mice with *Ripk3<sup>-/-</sup>* mice and assayed their BMDMs for inflammasome activity. *Tnfaip3<sup>-/-</sup>Ripk3<sup>-/-</sup>* double mutant BMDMs exhibited drastically reduced IL-1 $\beta$  secretion in response to poly(I:C), LPS, or Zym when compared to *Tnfaip3<sup>-/-</sup>* cells (Figure 2F). Thus, RIPK3 is critical for the spontaneous NLRP3 activity in A20-deficient cells.

#### A20 Restricts NLRP3 Activity Independently of Its Role in NF-κB Regulation

The expression of both pro-IL-1 $\beta$  (Figure 1B) and NLRP3 is induced by NF-κB activation (Bauernfeind et al., 2009). As A20 restricts TLR-induced NF-κB signaling (Boone et al., 2004; Turer et al., 2008), we determined whether the spontaneous NLRP3 inflammasome activity in A20-deficient cells results from increased pro-IL-1 $\beta$  or NLRP3 expression. We noted the amounts of pro-IL-1 $\beta$  expressed in WT and *Tnfaip*3<sup>-/-</sup> cells induced by various TLR ligands correlated poorly with the amounts of secreted mature IL-1 $\beta$  (Figures 1A and 1B). For example, poly(I:C) stimulation induced much less pro-IL-1ß expression than CpG or gardiquimod (Gdq) from  $Tnfaip3^{-/-}$  cells (Figure 1B); yet, poly(I:C) induced robust IL-1 $\beta$  secretion while Gdq and CpG did not (Figures 1A and 1C). More dramatically, poly(I:C) induced much less pro-IL-1 $\beta$  expression in *Tnfaip3<sup>-/-</sup>* cells than WT cells stimulated with Zym, LPS, Gdq, or CpG (Figure 1B); yet, poly(I:C)-stimulated Tnfaip3<sup>-/-</sup> cells readily processed pro-IL-1 $\beta$  for secretion while WT cells failed to do so with all TLR ligands tested (Figures 1A and 1C). We next assayed for NLRP3 expression in control and *Tnfaip3<sup>-/-</sup>* cells in response to various TLR ligands. The NLRP3 expression patterns paralleled those of pro-IL-1ß (Figure S1A). These data strongly suggest



#### Figure 2. Spontaneous NLRP3 Activity in *Tnfaip3<sup>-/-</sup>* Cells Depends on RIPK3

(A and B) As shown in (A), IL-1 $\beta$  responses by WT or *Tnfaip*3<sup>-/-</sup> BMDMs to ATP were measured by ELISA. Cells were pre-stimulated with LPS for 4 hr (IL-1 $\beta$  secretions displayed on left of graph), followed by 30 min pulse with 5 mM ATP. Three hr after addition of fresh media, IL-1 $\beta$  responses were measured (right of graph). (B) Pyroptotic responses to ATP were measured by LDH (lactate dehydrogenase) release assays.

(C-E) Effects of NLRP3, ASC, or Casp1/Casp11 deficiency on IL-1 $\beta$  secretion by *Tnfaip3<sup>-/-</sup>* cells were assessed. Single and compound mutants were stimulated with the indicated ligands for ~15 hr (C and D) or with 500 ng/mL LPS for the indicated time (E). The ligands used in (C) and (D) were 1 µg/mL Pam3CSK4 (P3CSK4), 100 µg/mL poly(I:C), 500 ng/mL LPS, 100 ng/mL flagellin (Flag), 3 µg/mL Gdq, and 5 µM CpG.

(F) Effects of RIPK3 deficiency on IL-1 $\beta$  responses by A20-deficient cells to poly(I:C), LPS, and Zym were assessed by ELISA. Each bar in graphs represents mean of triplicate wells + SD. \* denotes statistical significance (p < 0.05) when comparison is made between control and A20-deficient cells for each condition. # denotes statistical significance by Student's t test (p < 0.05) when comparison is made between A20 deficiency alone with the indicated compound mutations.  $\neq$  denotes statistically significant contribution by NLRP3, ASC, or Casp1 and Casp11 functions to spontaneous IL-1 $\beta$  secretion by A20-deficient cells.

that A20 regulates NLRP3 activity in a manner separate from regulating pro-IL-1 $\beta$  and NLRP3 transcription.

Both TRIF and MyD88 adaptor molecules participate in TLR4 signaling (Hoebe et al., 2003). Hence, we determined to what extent MyD88 deficiency might reduce the NLRP3 and pro-IL-1 $\beta$  expression in A20-deficient cells. Upon LPS stimulation, MyD88 deficiency decreased the amounts of NLRP3 and pro-IL-1 $\beta$  proteins in *Tnfaip3<sup>-/-</sup>Myd88<sup>-/-</sup>* cells to those of WT cells

(Figure S1B). Yet,  $Tnfaip3^{-/-}Myd88^{-/-}$  cells secreted mature IL-1 $\beta$  in response to LPS (Figure 1E), whereas WT cells did not (Figure 1A). These data suggest that A20 restrains a TRIF-dependent signal that can otherwise cause aberrant NLRP3 activation.

Unlike *Tnfaip3<sup>-/-</sup>* single mutant cells, *Tnfaip3<sup>-/-</sup>Ripk3<sup>-/-</sup>* compound mutant cells secreted little, if any, processed IL-1 $\beta$  (Figure 2F). Although RIPK3 has been reported to play no roles in TLR-induced transcriptional responses (Newton et al., 2004),



we entertained the possibility that RIPK3 deficiency might aberrantly affect the NF- $\kappa$ B-induced transcriptional responses in A20-deficient cells. We found the kinetics and magnitudes of LPS-induced I $\kappa$ B $\alpha$  phosphorylation and degradation to be similar between *Tnfaip3<sup>-/-</sup>* single mutant and *Tnfaip3<sup>-/-</sup> Ripk3<sup>-/-</sup>* compound mutant cells (Figure S1C). Moreover, *Tnfaip3<sup>-/-</sup> Ripk3<sup>-/-</sup>* cells expressed similar amounts of pro-IL-1 $\beta$  and NLRP3 as *Tnfaip3<sup>-/-</sup>* single mutant cells (Figure S1C). Taken together, these observations suggest that A20 inhibits a TRIF- and RIPK3-dependent step in NLRP3 inflammasome activation that is distinct from TLR-triggered transcription.

#### A20 Interacts with Inflammasome Proteins

Our observations that A20 restricts LPS-induced spontaneous NLRP3 activation independently of its inhibitory role in NF- $\kappa$ B signaling suggest that A20 might directly act on inflamma-

## Figure 3. A20 Associates with Pro-IL-1 $\beta$ Complex Consisting of Casp1, Casp8, RIPK1, and RIPK3

(A) BMDMs cultured from WT,  $Tnfaip3^{-/-}$ , or  $casp1/11^{-/-}$  mice were stimulated with 500 ng/mL LPS for 0–6 hr, lysed, and subjected to Casp1 IP, followed by IB analyses for A20 and pro-IL-1 $\beta$  interaction. Input for each IP is shown on the right. (B) Pro-IL-1 $\beta$  was IP'd from lysates of control and A20-deficient BMDMs stimulated with 500 ng/mL LPS for 0–12 hr. Co-association of each indicated protein with pro-IL-1 $\beta$  was analyzed by IB. Input IB analyses are shown on the right.

(C) Control and A20-deficient cells were stimulated with LPS for 6 hr, followed by 5 mM ATP for 30 min. Pro-IL-1 $\beta$  IPs were performed as in (B) to assay for changes in the interaction of each indicated protein with pro-IL-1 $\beta$ . To avoid potential complications by TNF- $\alpha$  autocrine signaling to the assays in (B) and (C), control and A20-deficient cells on *Tnf*<sup>-/-</sup> background were used. Similar results have been obtained using cells on *Tnf*<sup>+/+</sup> background. Arrows indicate cleaved RIPK1.

somes. To investigate whether A20 associates with inflammasome proteins, we IP'd Casp1 from BMDMs stimulated with LPS for 2-6 hr and immunoblotted for A20. Inducible interaction between Casp1 and A20 was evident upon LPS stimulation alone that was not detected in LPS-stimulated Casp1/11-/- BMDMs (Figure 3A). To further investigate A20 interaction with inflammasome proteins, we IP'd pro-IL-1ß from LPS-stimulated control and Tnfaip3<sup>-/-</sup> cells. Remarkably, A20 also co-IP'd with pro-IL-1ß from Tnfaip3+/+ lysates, with maximal association occurring 3 hr after LPS stimulation (Figure 3B). The presence of cleaved IL-1ß was also evident in the Tnfaip3<sup>-/-</sup> IPs (Figure 3B). As we observed exaggerated NLRP3 activities in A20-deficient BMDMs to LPS plus

ATP stimulation (Figures 2A and 2B), we asked whether ATP stimulation induces additional A20 recruitment to pro-IL-1 $\beta$ . We found that LPS-primed control cells recruited more A20 to pro-IL-1 $\beta$  within 15 min of ATP addition (Figure 3C). Blocking K<sup>+</sup> efflux had minimal effect on the A20-pro-IL-1 $\beta$  association, suggesting that A20 is recruited to pro-IL-1 $\beta$  prior to P2X7-induced NLRP3 activation. Hence, A20 interacts with pro-IL-1 $\beta$  after LPS stimulation, and NLRP3 inflammasome activation by ATP further enhances these interactions.

Unexpectedly, the IP experiments above also revealed Casp1pro-IL-1 $\beta$  pre-association in WT cells (Figures 3A, 3B, 3C), despite the absence of pro-IL-1 $\beta$  processing (Figure 1A). We also found Casp8, RIPK1, and RIPK3 to be present in this complex (Figures 3B and 3C). Since RIPK3 is necessary for the spontaneous secretion of IL-1 $\beta$  by A20-deficient cells (Figure 2F), these intriguing results suggest that (1) pro-IL-1 $\beta$  pre-associates

with Casp1 prior to inflammasome activation and (2) the absence of A20 allows for this complex to proceed with pro-IL-1ß processing in a RIPK3-dependent manner. We thus determined the functional consequences of A20 deficiency on this complex. More Casp1, Casp8, and RIPK1 were associated with pro-IL-1ß in A20-deficient cells (Figures 3B and 3C). A fraction of RIPK1 was cleaved in pro-IL-1ß complexes from A20-deficient cells, suggesting the presence of active Casp8 (Figure 3B, arrows), because RIPK1 is a Casp8 substrate (Humphries et al., 2014). This observation is consistent with the increased active (cleaved) Casp8 in the lysates (Figure 3B). Because Casp8 can cleave pro-IL-1β (Bossaller et al., 2012; Gringhuis et al., 2012; Gurung et al., 2014; Maelfait et al., 2008; Vince et al., 2012), the active Casp8 in this complex might be responsible for the residual IL-1 $\beta$  secretion by *Tnfaip3<sup>-/-</sup>Casp1/11<sup>-/-</sup>* cells (Figures 2C and 2E). This possibility was supported by our observation that this residual IL-1ß secretion was inhibited by the Casp8 inhibitor, Z-IETD-FMK (data not shown). Finally, ATP stimulation of either control or A20-deficient cells following LPS caused markedly more IL-1ß cleavage, but did not augment Casp1 or Casp8 association with pro-IL-1<sub>β</sub> (Figure 3C). Hence, ATP activates-and A20 inhibits-the activities of Casp1 and/or Casp8 molecules that are pre-bound to pro-IL-1 $\beta$ .

#### RIPK1 Catalytic Activity Promotes Spontaneous pro-IL-1β Processing in A20-Deficient Cells

RIPK3 activation usually requires the kinase activity of RIPK1 during programmed necrosis (Humphries et al., 2014). As we observed a requirement for RIPK3 in the spontaneous pro-IL-1ß processing by A20-deficient cells (Figure 2F), as well as the presence of RIPK1 in the pro-IL-1ß complex (Figures 3B. 3C, 4D), we asked whether RIPK1 activity is similarly required. Treatment of A20-deficient cells with LPS plus the RIPK1 inhibitor, necrostatin-1 (Nec1) (Degterev et al., 2008), reduced pro-IL-1β processing in a dose-dependent manner (Figure S2A). To determine whether the RIPK1 activity is accompanied by formation of RIPK1-RIPK3 complexes, or ripoptosomes (Feoktistova et al., 2011; Tenev et al., 2011), we IP'd RIPK1 from LPS-stimulated control and A20-deficient cells. More pro-IL-1ß was co-IP'd with RIPK1 from A20-deficient cells (Figure S2B), confirming the RIPK1-pro-IL-1β association observed by pro-IL-1ß IPs (Figures 3B and 3C). However, no increased RIPK1-RIPK3 interaction was observed regardless of LPS stimulation (Figure S2B). Taken together, our data suggest that A20 inhibits RIPK1 association with pro-IL-1 $\beta$  and that RIPK1 catalytic activity and RIPK3 drive spontaneous pro-IL-1ß processing in A20-deficient cells without ripoptosome formation.

## Pro-IL-1β Complex Contains K63-Linked and Unanchored Poly-Ub

Because A20 restricts NF- $\kappa$ B signaling by modifying Ub chains on key signaling proteins, we investigated whether inflammasome activation involves ubiquitination. We IP'd pro-IL-1 $\beta$  from LPS-stimulated WT or *Tnfaip3<sup>-/-</sup>* cells and probed for Ub. A high molecular weight complex containing Ub was evident in pro-IL-1 $\beta$  IPs from both WT and *Tnfaip3<sup>-/-</sup>* BMDMs (Figure 4A). Longer exposures of IL-1 $\beta$  immunoblot following pro-IL-1 $\beta$  IPs also revealed that pro-IL-1 $\beta$  itself is modified by high molecular weight moieties with laddering patterns suggestive of ubiquitination (Figure 4A, middle panel). Strikingly, ATP robustly augmented the formation of this Ub-containing complex in both control and A20-deficient cells (Figure 4B, left panel, 3<sup>rd</sup> and 7<sup>th</sup> lanes), suggesting that NLRP3 inflammasomes are ubiquitinated. Moreover, the pro-IL-1 $\beta$  complex ubiquitination was markedly increased in A20-deficient cells (Figure 4A), and the timing of pro-IL-1 $\beta$ -associated Ub signals in A20-deficient cells correlated with the temporal increase in IL-1 $\beta$  secretion (Figure 1D). Preventing K<sup>+</sup> efflux had no effects on the ATP-induced ubiquitination (Figure 4B, left panel, 4<sup>th</sup> and 8<sup>th</sup> lanes), indicating that the poly-Ub chains are formed prior to NLRP3 oligomerization. Thus, these observations collectively suggest that A20 restricts pro-IL1 $\beta$  complex ubiquitination prior to NLRP3-ASC inflammasome assembly.

To better characterize the pro-IL-1ß complex ubiquitination in LPS-stimulated A20-deficient cells, we utilized linkage-specific de-ubiquitinases (DUBs) to determine the types of Ub chains present in the pro-IL-1β complex (Mevissen et al., 2013). We first treated pro-IL-1ß IPs from these cells with the non-specific DUB USP2 (ubiquitin carboxyl-terminal hydrolase 2). USP2 eliminated all immunoreactive Ub from the pro-IL-1 $\beta$  IP, confirming that our anti-Ub immunoblots detected ubiquitination (Figure 4C, last lane). Much of the covalent, high molecular weight modifications on pro-IL-1ß were also eliminated by USP2 treatment, reinforcing the notion that pro-IL-1B itself is modified by poly-Ub (Figure 4C). To determine whether the pro-IL-1β-associated Ub chains contain non-degradative Ub linkages, we treated the pro-IL-1ß IPs with the K63-specific DUB AMSH (associated molecule with a Src homology 3 domain of signal transducing adaptor molecule) and CyID (Cylindromatosis), which cleaves both linear and K63-linked chains (Komander et al., 2008; 2009). Both AMSH and CyID reduced the pro-IL-1β-associated poly-Ub (Figure 4C, 2<sup>nd</sup> and 3<sup>rd</sup> lanes), indicating the presence of K63-linked Ub chains in the pro-IL-1ß complex. To determine whether the ubiquitin chains in this complex are anchored, we treated the pro-IL-1ß IP with isopeptidase T (IsoT), a DUB specific for unanchored chains (Wilkinson et al., 1995). IsoT also reduced the pro-IL-1 $\beta$ -associated ubiquitin (Figure 4C, 4<sup>th</sup> lane). Thus, the ubiquitin chains associated with pro-IL-1ß include unanchored, as well as K63-linked, poly-Ub, both types known to support signal propagation by encouraging nondegradative protein-protein interactions (Corn and Vucic, 2014; Xia et al., 2009).

We next determined whether K48 Ub linkages exist in the complex by probing the DUB-treated pro-IL-1 $\beta$  IPs with an anti-K48-linked poly-Ub antibody. Without DUB treatment, the presence of K48-linked poly-Ub in the pro-IL-1 $\beta$  complex was clearly evident (Figure 4C, 1<sup>st</sup> lane). AMSH treatment had no effects on the K48-linked Ub signals, confirming its specificity for K63 linkages (Figure 4C, 2<sup>nd</sup> lane). Interestingly, IsoT treatment removed much of the K48-linked Ub signals, indicating that the majority of K48 linkages are present in unanchored chains (Figure 4C, 4<sup>th</sup> lane). It has been reported that K48 linkages in unanchored poly-Ub or mixed chains can also support positive roles in signaling independently of proteasomal degradation (Jiang et al., 2012; Rajsbaum et al., 2014). Hence, the pro-IL-1 $\beta$  complex is ubiquitinated with chains that can support non-proteasomal signal propagation.



#### Figure 4. Pro-IL-1β in A20-Deficient Cells Associates with RIPK3-Dependent K63-Linked and Unanchored Poly-Ub

(A) WT and *Tnfaip*3<sup>-/-</sup> BMDMs were stimulated with 500 ng/mL LPS for 0–7 hr. Pro-IL-1β IPs were performed as in Figure 3B and analyzed for Ub signals and Casp1 interaction. Input IB analyses are shown on the right.

(B) Control and A20-deficient cells on  $Tnf^{-/-}$  background were primed with 500 ng/mL LPS for 6 hr and subsequently stimulated with 5 mM ATP for 15–20 min. Pro-IL-1 $\beta$  IPs, followed by IB analyses, were performed as in Figure 3C. IB analyses of each reaction input are shown on the right.

(C) Pro-IL-1β IPs from A20-deficient BMDMs stimulated with 500 ng/mL LPS for 6 hr were subjected to DUB proteolysis. Ub signals were subsequently analyzed by IB.

(D) Control or A20-deficient BMDMs with or without RIPK3 deficiency were stimulated with 500 ng/mL LPS for 6 hr. Subsequently, Pro-IL-1β IPs, followed by IB analyses, were performed to assay for Ub signals and the interaction of each indicated protein with pro-IL-1β. Arrow indicates cleaved RIPK1. Note the anti-IL-1β antibody used in IB analyses cannot recognize IL-1β that is modified by >10 Ub molecules, perhaps due to steric hindrance by the Ub modification.



## $\label{eq:pro-IL-1} \begin{array}{l} \beta \text{-} Associated \ Ubiquitination \ Depends \ on \ RIPK3, \\ But \ Not \ NLRP3, \ ASC, \ or \ Casp1 \end{array}$

Our data above suggest that ubiquitination of pro-IL-1ß complex correlates with inflammasome activity. As RIPK3 deficiency abolished most of IL-1ß secretion by A20-deficient cells, we next determined whether rendering Tnfaip3<sup>-/-</sup> cells RIPK3 deficient would similarly affect the pro-IL-1β-associated Ub signals. Strikingly, Tnfaip3<sup>-/-</sup>Ripk3<sup>-/-</sup> cells displayed markedly less ubiquitination of pro-IL-1ß complex than Tnfaip $3^{-/-}$ Ripk $3^{+/+}$  cells after LPS stimulation (Figure 4D). Of note, Tnfaip3<sup>-/-</sup>Ripk3<sup>-/-</sup> cells also displayed reduced Casp8 activity, as indicated by the decreased presence of cleaved Casp8, as well as its putative substrate, RIPK1 (Figure 4D, right panel, arrow). To determine whether formation of this ubiquitinated complex required NLRP3, ASC, or Casp1, we tested Tnfaip3<sup>-/-</sup>NIrp3<sup>-/-</sup>, Tnfaip3<sup>-/-</sup>Asc<sup>-/-</sup>, and Tnfaip3<sup>-/-</sup> Casp1/11-/- compound mutant cells in a similar fashion. In contrast to Tnfaip3-/-Ripk3-/- cells, ubiquitination of pro-IL-1β complex in these compound mutant cells resembled those seen in single mutant  $Tnfaip3^{-/-}$  cells (Figures S3A and S3C). Furthermore, ATP stimulation of these compound mutant cells induced similar amounts of Ub modification on pro-IL-1ß complexes (Figures S3A and S3B). Taken together, these data suggest that exaggerated ubiquitination of pro-IL-1ß complexes in A20-deficient cells occurs downstream of RIPK3 but upstream of NLRP3, ASC, and Casp1/Casp11 inflammasome assembly.

### Figure 5. Overexpression of A20 Suppresses Casp1 Activity

(A) 293T cells seeded in 6-well plates were transiently transfected with 150 ng/well of each plasmid encoding HA-Casp1, Myc-ASC, or pro-IL-1 $\beta$  linked to GFP via an IRES sequence, plus 0-50 ng/well of plasmid encoding Flag-A20. 24 hr later, IL-1 $\beta$  secretions in the supernatant were quantified by ELISA (upper left panel) and analyzed by IB following IL-1 $\beta$  IP (lower left panel). IB analyses of the transfected cell lysates are shown on the right.

(B) 293T cells seeded in 6-cm dishes were transfected with 500 ng/well of each construct for Streptag-Casp1, Myc-ASC, and pro-IL-1 $\beta$  linked to GFP via an IRES sequence. 6 µg of plasmid encoding HA-tagged WT or K63-only Ub was also included along with 0-50 ng/well of Flag-A20 construct. 24 hr later, cells were lysed, subjected to pro-IL-1 $\beta$  IP, and analyzed by IB for the indicated proteins. Input immunoblots are shown on the right.

#### A20 Suppresses Casp1 Activity

Our data above suggest that A20 might act directly on inflammasome complexes to prevent their spontaneous activity. To test whether A20 can inhibit Casp1 activity independently of TLR signaling, 293T cells were transiently transfected with expression plasmids of ASC, Casp1, and pro-IL-1 $\beta$  coupled to GFP by an IRES sequence, with or

without A20. As previously reported (Srinivasula et al., 2002), overexpression of ASC and Casp1 led to their self assembly, and the resultant Casp1 activity was evident through the secretion of mature IL-1B (Figure 5A). Importantly, co-expression of A20 dampened this process in a dose-dependent manner (Figure 5A, left panels) without affecting GFP or pro-IL-1β expression (Figure 5A, right panels). These results reinforce the point that A20 can suppress pro-IL-1ß processing independently of the amount of pro-IL-1ß expressed. We next determined whether this Casp1 activity involves ubiguitination by including constructs for the expression of HAtagged WT Ub, as well as a mutant that can undergo only K63-linked conjugation. As observed with BMDMs, K63-linked Ub signals were detected in pro-IL-1ß IPs, and overexpression of A20 dampened this signal in a dose-dependent manner (Figure 5B). These results reinforce our data above that A20 restricts K63-linked ubiquitination of the pro-IL-1ß complex (Figure 4C).

## Ubiquitination of pro-IL-1 $\beta$ Supports Casp1 Proteolytic Processing

Our finding that pro-IL-1 $\beta$ -associated ubiquitination in *Tnfaip3<sup>-/-</sup>* cells was not eliminated by NLRP3, ASC, or Casp1/11 deficiency (Figures S3A–S3C) suggest that pro-IL-1 $\beta$  itself might undergo ubiquitination. This notion is also supported by our observation that pro-IL-1 $\beta$  in activated macrophages is modified by high molecular weight moieties, which



### Figure 6. Lys133 of pro-IL-1β Supports Ubiquitination and Processing

(A) *Tnfaip3<sup>-/-</sup>* BMDMs stimulated with 500 ng/mL LPS for 6 hr were subjected to mass spectrometric analyses. CID tandem mass spectrum was obtained from a precursor ion with m/z value 759.0690<sup>+3</sup>, present in the pull-downs of K-e-GG peptides from the trypsin-digested lysates. Database search identifies it as the peptide expanding amino acids 127 to 144 of pro-IL-1 $\beta$ , carrying a diglycine remnant on K133 (theoretical mono-isotopic m/z = 759.0691<sup>+3</sup>). Expectation value for the sequence assignment is 8.1 × 10<sup>-5</sup>. Sequence ions are labeled in the figure and indicated with marks over (C-terminal ions) and below (N-terminal ions) the sequence of the peptide.

(B and C) 293T cells seeded in 6-well plates were transiently transfected with 500 ng/well of Myc-ASC expression plasmid, plus 500 ng/well of construct encoding either WT or mutant pro-IL-1 $\beta$  linked to GFP via an IRES sequence. IL-1 $\beta$  secretion in each condition was subsequently measured by ELISA (B). C284S mutant form of Casp1 activity. The noncleavable D117A pro-IL-1 $\beta$  mutant was additionally included as a negative control. (C) IB analyses of the cells transfected in (B) were performed to illustrate equal transfection in the conditions.

(D) 293T cells seeded in 6-well plates were transfected with 500 ng/well of each construct for Myc-ASC, Streptag-Casp1, and WT or K133R pro-IL-1 $\beta$  linked to GFP via IRES. 3 µg of expression plasmid encoding HA-tagged WT, K48R, or K63R Ub was also included. 24 hr later, cells were lysed and subjected to pro-IL-1 $\beta$  IP followed by IB analyses for Ub signals and Casp1 association. Input immunoblots are shown below.

cessing (Figures 6B and 6C). K133R pro-IL-1 $\beta$  protein also exhibited less total, non-K48, and non-K63 ubiquitination than WT pro-IL-1 $\beta$  (Figure 6D). By contrast, the non-cleavable D117A mutant form of pro-IL-1 $\beta$  exhibited no obvious defects in ubiquitination (Figure 6D).

were removed by USP2 (Figures 4A-4C). We thus utilized anti-G-G antibody-assisted mass spectrometry to find potential ubiquitinated IL-1 $\beta$  peptides from LPS-stimulated *Tnfaip3<sup>-/-</sup>* BMDMs. These analyses revealed that an evolutionarily conserved K133 of mouse pro-IL-1ß undergoes ubiquitination (Figure 6A). As these studies were performed on endogenous proteins in primary cells undergoing LPS stimulation, and as K133 ubiquitination was detected repeatedly, the ubiquitination at this site is likely to be a dominant and physiological event. To investigate the functional impact of pro-IL<sub>β</sub> ubiquitination, we generated a K133R mutant of pro-IL-1 $\beta$  and tested its processing in 293T cells. Mutant K133R pro-IL-1ß was cleaved less efficiently than WT protein, leading to less secretion of mature IL-1ß (Figures 6B and 6C). As expected, mutating the cleavage site of pro-IL-1ß at D117 as well as the catalytic site of Casp1 at C284 abolished pro-IL-1ß pro-

DISCUSSION

In this study, we have uncovered a role for A20 in restricting NLRP3 inflammasome functions. A20-deficient macrophages exhibit spontaneous NLRP3 activity to various stimuli that are otherwise insufficient to induce pro-IL-1 $\beta$  processing in normal cells. Interestingly, TLRs that only utilize MyD88 to signal did not trigger this response. AIM2 (absent in melanoma 2) inflammasomes response to poly(dA:dT) in *Tnfaip3<sup>-/-</sup>* cells were also normal following TLR2 or TLR7 priming (Figures S4A and S4B). Hence, A20 might be particularly important for preventing spontaneous NLRP3 activation by receptors that share common components with TLR3 signaling. The

Thus, these data support the notion that pro-IL-1β ubiquitina-

tion at K133 promotes its proteolytic cleavage.

absence of DAMPs in the supernatants of stimulated  $Tnfaip3^{-/-}$  cells suggests an intrinsic mechanism of NLRP3 activation.

We previously described an important role for A20 in restricting TLR-induced NF- $\kappa$ B signaling (Boone et al., 2004; Turer et al., 2008), and a recent study showed that exaggerated TLR-induced expression of inflammasome proteins causes arthritis in mice lacking A20 in myeloid cells (Vande Walle et al., 2014). Our current study, however, reveals that the spontaneous NLRP3 activity in A20-deficient cells does not correlate entirely with the amount of NF- $\kappa$ B-induced pro-IL-1 $\beta$  or NLRP3 protein. Hence, A20 likely restricts NLRP3 inflammasome activity via multiple mechanisms.

Genetic defects that lead to spontaneous IL-1 $\beta$  secretion by innate immune cells have been reported in three other instances. In one case, ablation of the Atg16L1 gene involved in autophagy resulted in spontaneous NLRP3 activation by LPS alone (Saitoh et al., 2008). In another, BMDMs from mice rendered deficient in all three IAPs similarly exhibited spontaneous NLRP3 inflammasome activity (Vince et al., 2012). Most recently, loss of second signal requirement for pro-IL-1ß processing was reported for Casp8-deficient DCs (Kang et al., 2013). The molecular mechanisms by which Tnfaip3<sup>-/-</sup> cells acquire spontaneous NLRP3 activity are likely to be distinct from these reported studies. First, A20 deficiency exaggerates, not blunts, autophagic response to LPS (Shi and Kehrl, 2010a; 2010b). Second, as observed here and previously reported (Jin et al., 2009), Tnfaip3<sup>-/-</sup> cells exhibit increased Casp8 activity. While the spontaneous NLRP3 activity of Tnfaip3<sup>-/-</sup> cells resembles that of the IAP triple mutants (Vince et al., 2012), marked differences in their responses are also noted. As in IAP triple-deficient cells, RIPK3 may drive NLRP3 inflammasome activation in Tnfaip3<sup>-/-</sup> cells via ROS generation (data not shown). However, unlike the IAP triple mutants, which exhibit spontaneous pro-IL-1β processing in response to all TLR ligands, A20-deficient cells selectively display this response after TLR signals that utilize TRIF. Moreover, we found no evidence for increased formation of RIPK1-RIPK3 complexes in Tnfaip3<sup>-/-</sup> cells after LPS stimulation. Whereas RIPK1 catalytic activity is dispensable in IAP triple mutants, it appears necessary for the spontaneous NLRP3 activity in Tnfaip3<sup>-/-</sup> cells. Thus, our studies build on the concept that RIPK3 supports NLRP3 inflammasome activation, but identify a pathway toward this outcome that is inhibited by A20.

Using pro-IL-1 ß IPs, we have discovered that A20 exists in a complex that contains Casp1, Casp8, RIPK1, and RIPK3 in LPS-stimulated BMDMs. These protein interactions appear specific because (1) pull-downs using the isotype control antibody failed to co-IP these proteins and (2) pro-IL-1ß IPs from lysates of unstimulated cells, which do not express any pro-IL-1 $\beta$ , yielded little, if any, co-associated proteins. These results suggest two potential mechanisms by which A20 suppresses NLRP3 inflammasomes. First, the increased amounts of Casp1, Casp8, and RIPK1 associating with pro-IL-1 $\beta$  in A20deficient cells indicate that A20 regulates the composition of this complex. Second, as ubiquitination of pro-IL-1ß complex is increased in A20-deficient cells in response to LPS alone or LPS plus ATP, these Ub chains might support pro-IL-1ß processing and subsequent secretion. Neither ASC deficiency (data not shown) nor preventing K<sup>+</sup> efflux affected the composition or ubiquitination of the pro-IL-1 $\beta$  complex. Thus, both ubiquitination and formation of this complex occur prior to NLRP3-ASC inflammasome assembly.

We have observed increased Casp8 activity in A20-deficient cells that tightly correlates with spontaneous IL-1 $\beta$  secretion. Exaggerated Casp8 activity in LPS-stimulated A20-deficient BMDMs might reflect A20's ability to restrict Casp8 (Jin et al., 2009). As Casp8 can cleave pro-IL-1 $\beta$  in the absence of Casp1 (Bossaller et al., 2012; Gringhuis et al., 2012; Gurung et al., 2014; Maelfait et al., 2008; Vince et al., 2012), the observed residual IL-1 $\beta$  secretions by *Tnfaip3<sup>-/-</sup>Casp1/11<sup>-/-</sup>* cells might reflect pro-IL-1 $\beta$  processing by Casp8. Additionally, abolishing RIPK3 in A20-deficient cells reduced Casp8 activity, as well as pro-IL-1 $\beta$  processing. A recent finding suggests that Casp8 activity can prime and activate NLRP3 inflammasomes (Gurung et al., 2014). It is thus tempting to speculate that Casp8 activity initiates NLRP3 and Casp1 activation in A20-deficient cells.

We have discovered the presence of K63-linked and unanchored poly-Ub in the pro-IL1 $\beta$  complex, both chain types known to play critical roles in signal propagation (Corn and Vucic, 2014; Xia et al., 2009). While K48-linked poly-Ub is also present, the majority of K48 linkages appeared to be associated with unanchored chains. Recently, K48 linkages in unanchored or mixed Ub chains have been reported to propagate signaling independently of the proteasomes (Jiang et al., 2012; Rajsbaum et al., 2014). Thus, the presence of K63 linkages (and potentially, K48 linkages in unanchored chains) in pro-IL1 $\beta$  complex suggests that NLRP3 inflammasome activation involves ubiquitination, a step that A20 inhibits.

NLRP3, ASC, and Casp1 deficiency had no effects on the pro-IL-1β-associated Ub signals in LPS-stimulated A20-deficient cells. These observations indicate that this ubiquitination event is distinct from those previously reported for Casp1 and ASC during NLRP3 inflammasome activation (Labbé et al., 2011; Rodgers et al., 2014). Our IP/IB, mass-spectrometry, and mutational analyses revealed that K133 of mouse pro-IL-1 $\beta$  is ubiquitinated and that ubiquitination of this site supports pro-IL-1ß proteolytic cleavage. IL-1ß ubiquitination might support pro-IL-1ß cleavage by encouraging higher order oligomerization of inflammasome components; future studies will be needed to define this mechanism more precisely. Given the known roles of ubiquitination in autophagy (Shaid et al., 2013), IL-1β ubiquitination could also potentially support IL-1ß secretion through an autophagy-based unconventional secretory pathway (Dupont et al., 2011).

In summary, we have discovered that A20 plays a critical role in preventing spontaneous NLRP3 inflammasome activation and in limiting its activity by exogenous stimuli. Given the genetic links of A20 to multiple human inflammatory diseases, our findings establish links between A20, ubiquitination, inflammasomes, and human diseases.

#### EXPERIMENTAL PROCEDURES

#### Mice

*Tnfaip3<sup>-/-</sup>*, *NIrp3<sup>-/-</sup>*, *Asc<sup>-/-</sup>*, *Casp1/11<sup>-/-</sup>*, *Tnf<sup>-/-</sup>*, and *Ripk3<sup>-/-</sup>* mutant mice have been previously described. All mice used in this study were housed and bred in a specific pathogen-free facility according to the IACUC guidelines of UCSF.

#### **BMDM Cultures and Treatments**

Primary BMDMs were cultured from mouse femurs and tibia using standard protocols described in Supplemental Experimental Procedures. For ELISAs, cells were plated at 1–2 million cells in 0.5 ml per well in 24-well format. Innate immune receptor ligands were purchased from InvivoGen. Anti-CD40 antibody, clone FGK4.5, was produced by the UCSF antibody core facility. Recombinant murine TNF- $\alpha$  and Necrostatin-1 were purchased from R&D Systems and Enzo Life Sciences, respectively. Supernatants were typically harvested between 2–18 hr post-stimulation. To induce NLRP3 activation by ATP, we primed BMDMs with LPS at 50–500 ng/mL for 4–16 hr and subsequently treated with PBS containing 5 mM ATP for 15–30 min as described in Supplemental Experimental Procedures.

#### **Protein Overexpression Analyses**

293T cells were grown to ~70%–80% confluence in DMEM containing 10% FBS, Pen-Strep, and L-Glut before transfection using Lipofectamine LTX without Plus Reagent (Invitrogen). The following full-length cDNA expression plasmids were either generated using commercially available vectors or purchased from the indicated vendors: Myc-DDK tagged ASC in pCMV6-Entry (Origene); N-terminally HA-tagged Casp1 in pCMV-HA (Clonetech); N-terminally StrepTagged mouse Casp1 in pCMV-Script (Stratagene); Fullength IL-1 $\beta$  in pIRES2-ACGFP (Clontech); N-terminally Flag-tagged A20 in pCMV-Tag2 (Stratagene). 3× HA-tagged WT Ub, K63-only Ub, K48R Ub, and K63R Ub pcDNA3.1 constructs were kind gifts from Dr. Vishva Dixit (Genentech). Cells were typically transfected in 6-well format in 2 ml media with 150 ng/well of each vector, except for Ub constructs (which were used at 3 µg/well) and A20 (which varied from 0 to 50 ng/well).

#### **ELISAs and LDH Release Assays**

 $IL-1\beta$  (BD Biosciences) and IL-18 (eBioscience) ELISAs and LDH release assays (Cytotoxicity Detection Kit, Roche Life Science) were performed per manufacturer's instructions.

#### Immunoblotting and Immunoprecipitation

IB and IP analyses were performed using standard protocols described in Supplemental Experimental Procedures.

#### **DUB** Assays

Five 10-cm plates of *Tnfaip3<sup>-/-</sup>* cells (corresponding to 2 × 10<sup>8</sup> cells) were stimulated with 500 ng/mL LPS for 6 hr. Cells were lysed, and IL-1 $\beta$  IPs were performed in five separate tubes as described in Supplemental Experimental Procedures. During the last wash, the beads containing IL-1 $\beta$ -associated Ub complex were combined, split into several equal aliquots, and subjected to DUB treatments using protocols similar to those included in the UbiCREST kit (Boston Biochem) as detailed in Supplemental Experimental Procedures.

#### **Pro-IL-1**β Ubiquitination Site Determination

Day-6 *Tnfaip3<sup>-/-</sup>* BMDMs were seeded in twenty 15-cm plates and stimulated with 500 ng/mL LPS for 6 hr as described above. Subsequently, the cells were lifted using PBS containing 5 mM EDTA, pooled, centrifuged, and snap-frozen upon removal of PBS. The frozen cells were then subjected to lysis, followed by anti-Gly-Gly immunoprecipitation and UbiScan-assisted mass spectrometric analyses as detailed in Supplemental Experimental Procedures.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi. org/10.1016/j.immuni.2014.12.031.

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#### REFERENCES

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., et al. (2009). Cutting edge: NF- $\kappa$ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. Journal of Immunology *183*, 787–791.

Boone, D.L., Turer, E.E., Lee, E.G., Ahmad, R.-C., Wheeler, M.T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O., et al. (2004). The ubiquitinmodifying enzyme A20 is required for termination of Toll-like receptor responses. Nat. Immunol. *5*, 1052–1060.

Bossaller, L., Chiang, P.-I., Schmidt-Lauber, C., Ganesan, S., Kaiser, W.J., Rathinam, V.A.K., Mocarski, E.S., Subramanian, D., Green, D.R., Silverman, N., et al. (2012). Cutting edge: FAS (CD95) mediates noncanonical IL-1 $\beta$  and IL-18 maturation via caspase-8 in an RIP3-independent manner. Journal of Immunology *189*, 5508–5512.

Brown, G.D., Taylor, P.R., Reid, D.M., Willment, J.A., Williams, D.L., Martinez-Pomares, L., Wong, S.Y.C., and Gordon, S. (2002). Dectin-1 is a major betaglucan receptor on macrophages. J. Exp. Med. *196*, 407–412.

Catrysse, L., Vereecke, L., Beyaert, R., and van Loo, G. (2014). A20 in inflammation and autoimmunity. Trends Immunol. *35*, 22–31.

Chu, Y., Vahl, J.C., Kumar, D., Heger, K., Bertossi, A., Wójtowicz, E., Soberon, V., Schenten, D., Mack, B., Reutelshöfer, M., et al. (2011). B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice. Blood *117*, 2227–2236.

Corn, J.E., and Vucic, D. (2014). Ubiquitin in inflammation: the right linkage makes all the difference. Nat. Struct. Mol. Biol. *21*, 297–300.

Davis, B.K., Wen, H., and Ting, J.P.-Y. (2011). The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu. Rev. Immunol. *29*, 707–735.

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I.L., Korkina, O., Teng, X., Abbott, D., Cuny, G.D., Yuan, C., Wagner, G., et al. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. *4*, 313–321.

Dupont, N., Jiang, S., Pilli, M., Ornatowski, W., Bhattacharya, D., and Deretic, V. (2011). Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. EMBO J. *30*, 4701–4711.

Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D.P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G., and Leverkus, M. (2011). cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol. Cell *43*, 449–463.

Gringhuis, S.I., Kaptein, T.M., Wevers, B.A., Theelen, B., van der Vlist, M., Boekhout, T., and Geijtenbeek, T.B.H. (2012). Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1 $\beta$  via a noncanonical caspase-8 inflammasome. Nat. Immunol. *13*, 246–254.

Gurung, P., Anand, P.K., Malireddi, R.K.S., Vande Walle, L., Van Opdenbosch, N., Dillon, C.P., Weinlich, R., Green, D.R., Lamkanfi, M., and Kanneganti, T.-D. (2014). FADD and caspase-8 mediate priming and activation of the canonical and noncanonical NIrp3 inflammasomes. Journal of Immunology *192*, 1835–1846.

Hammer, G.E., Turer, E.E., Taylor, K.E., Fang, C.J., Advincula, R., Oshima, S., Barrera, J., Huang, E.J., Hou, B., Malynn, B.A., et al. (2011). Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. Nat. Immunol. *12*, 1184–1193.

He, S., Liang, Y., Shao, F., and Wang, X. (2011). Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc. Natl. Acad. Sci. USA *108*, 20054–20059.

Heger, K., Fierens, K., Vahl, J.C., Aszodi, A., Peschke, K., Schenten, D., Hammad, H., Beyaert, R., Saur, D., van Loo, G., et al. (2014). A20-deficient mast cells exacerbate inflammatory responses in vivo. PLoS Biol. *12*, e1001762.

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S.O., Goode, J., Lin, P., Mann, N., Mudd, S., et al. (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature *424*, 743–748.

Humphries, F., Yang, S., Wang, B., and Moynagh, P.N. (2014). RIP kinases: key decision makers in cell death and innate immunity. Cell Death Differ. Published online August 22, 2014. http://dx.doi.org/10.1038/cdd.2014.126.

Jiang, X., Kinch, L.N., Brautigam, C.A., Chen, X., Du, F., Grishin, N.V., and Chen, Z.J. (2012). Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. Immunity *36*, 959–973.

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R., and Ashkenazi, A. (2009). Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell *137*, 721–735.

Kaiser, W.J., Sridharan, H., Huang, C., Mandal, P., Upton, J.W., Gough, P.J., Sehon, C.A., Marquis, R.W., Bertin, J., and Mocarski, E.S. (2013). Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. J. Biol. Chem. *288*, 31268–31279.

Kang, T.-B., Yang, S.-H., Toth, B., Kovalenko, A., and Wallach, D. (2013). Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. Immunity *38*, 27–40.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., et al. (2011). Non-canonical inflammasome activation targets caspase-11. Nature 479, 117–121.

Komander, D., Lord, C.J., Scheel, H., Swift, S., Hofmann, K., Ashworth, A., and Barford, D. (2008). The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module. Mol. Cell *29*, 451–464.

Komander, D., Reyes-Turcu, F., Licchesi, J.D.F., Odenwaelder, P., Wilkinson, K.D., and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep. *10*, 466–473.

Kool, M., van Loo, G., Waelput, W., De Prijck, S., Muskens, F., Sze, M., van Praet, J., Branco-Madeira, F., Janssens, S., Reizis, B., et al. (2011). The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity. Immunity 35, 82–96.

Labbé, K., McIntire, C.R., Doiron, K., Leblanc, P.M., and Saleh, M. (2011). Cellular inhibitors of apoptosis proteins cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. Immunity 35, 897–907.

Lamkanfi, M., and Dixit, V.M. (2012). Inflammasomes and their roles in health and disease. Annu. Rev. Cell Dev. Biol. 28, 137–161.

Lee, E.G., Boone, D.L., Chai, S., Libby, S.L., Chien, M., Lodolce, J.P., and Ma, A. (2000). Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science *289*, 2350–2354.

Leemans, J.C., Cassel, S.L., and Sutterwala, F.S. (2011). Sensing damage by the NLRP3 inflammasome. Immunol. Rev. 243, 152–162.

Ma, A., and Malynn, B.A. (2012). A20: linking a complex regulator of ubiquitylation to immunity and human disease. Nat. Rev. Immunol. 12, 774–785.

Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S., and Beyaert, R. (2008). Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8. J. Exp. Med. *205*, 1967–1973.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., and Dixit, V.M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440, 228–232.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol. Cell *10*, 417–426.

Martinon, F., Mayor, A., and Tschopp, J. (2009). The inflammasomes: guardians of the body. Annu. Rev. Immunol. 27, 229–265.

Matmati, M., Jacques, P., Maelfait, J., Verheugen, E., Kool, M., Sze, M., Geboes, L., Louagie, E., Mc Guire, C., Vereecke, L., et al. (2011). A20 (TNFAIP3) deficiency in myeloid cells triggers erosive polyarthritis resembling rheumatoid arthritis. Nat. Genet. *43*, 908–912.

Mevissen, T.E.T., Hospenthal, M.K., Geurink, P.P., Elliott, P.R., Akutsu, M., Arnaudo, N., Ekkebus, R., Kulathu, Y., Wauer, T., El Oualid, F., et al. (2013). OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. Cell *154*, 169–184.

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B.L., Rajendiran, T.M., and Núñez, G. (2013). K<sup>+</sup> efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 38, 1142–1153.

Newton, K., Sun, X., and Dixit, V.M. (2004). Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol. Cell. Biol. *24*, 1464–1469.

Rajsbaum, R., Versteeg, G.A., Schmid, S., Maestre, A.M., Belicha-Villanueva, A., Martínez-Romero, C., Patel, J.R., Morrison, J., Pisanelli, G., Miorin, L., et al. (2014). Unanchored K48-linked polyubiquitin synthesized by the E3-ubiquitin ligase TRIM6 stimulates the interferon-IKK $\varepsilon$  kinase-mediated antiviral response. Immunity *40*, 880–895.

Rathinam, V.A.K., Vanaja, S.K., Waggoner, L., Sokolovska, A., Becker, C., Stuart, L.M., Leong, J.M., and Fitzgerald, K.A. (2012). TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. Cell *150*, 606–619.

Rodgers, M.A., Bowman, J.W., Fujita, H., Orazio, N., Shi, M., Liang, Q., Amatya, R., Kelly, T.J., Iwai, K., Ting, J., and Jung, J.U. (2014). The linear ubiquitin assembly complex (LUBAC) is essential for NLRP3 inflammasome activation. J. Exp. Med. *211*, 1333–1347.

Saitoh, T., Fujita, N., Jang, M.H., Uematsu, S., Yang, B.-G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., et al. (2008). Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.

Shaid, S., Brandts, C.H., Serve, H., and Dikic, I. (2013). Ubiquitination and selective autophagy. Cell Death Differ. *20*, 21–30.

Shembade, N., and Harhaj, E.W. (2012). Regulation of NF- $\kappa$ B signaling by the A20 deubiquitinase. Cell. Mol. Immunol. 9, 123–130.

Shi, C.-S., and Kehrl, J.H. (2010a). Traf6 and A20 differentially regulate TLR4-induced autophagy by affecting the ubiquitination of Beclin 1. Autophagy 6, 986–987.

Shi, C.-S., and Kehrl, J.H. (2010b). TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced autophagy. Sci. Signal. *3*, ra42.

Srinivasula, S.M., Poyet, J.-L., Razmara, M., Datta, P., Zhang, Z., and Alnemri, E.S. (2002). The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. J. Biol. Chem. 277, 21119–21122.

Strowig, T., Henao-Mejia, J., Elinav, E., and Flavell, R. (2012). Inflammasomes in health and disease. Nature 481, 278–286.

Tavares, R.M., Turer, E.E., Liu, C.L., Advincula, R., Scapini, P., Rhee, L., Barrera, J., Lowell, C.A., Utz, P.J., Malynn, B.A., and Ma, A. (2010). The ubiquitin modifying enzyme A20 restricts B cell survival and prevents autoimmunity. Immunity *33*, 181–191.

Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K., and Meier, P. (2011). The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol. Cell *43*, 432–448.

Turer, E.E., Tavares, R.M., Mortier, E., Hitotsumatsu, O., Advincula, R., Lee, B., Shifrin, N., Malynn, B.A., and Ma, A. (2008). Homeostatic MyD88-dependent signals cause lethal inflamMation in the absence of A20. J. Exp. Med. *205*, 451–464.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., and Aderem, A. (1999). The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature *401*, 811–815.

66 Immunity 42, 55–67, January 20, 2015 ©2015 Elsevier Inc.

Vande Walle, L., Van Opdenbosch, N., Jacques, P., Fossoul, A., Verheugen, E., Vogel, P., Beyaert, R., Elewaut, D., Kanneganti, T.D., van Loo, G., and Lamkanfi, M. (2014). Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. Nature *512*, 69–73.

Vereecke, L., Sze, M., Mc Guire, C., Rogiers, B., Chu, Y., Schmidt-Supprian, M., Pasparakis, M., Beyaert, R., and van Loo, G. (2010). Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis. J. Exp. Med. *207*, 1513–1523.

Verstrepen, L., Verhelst, K., van Loo, G., Carpentier, I., Ley, S.C., and Beyaert, R. (2010). Expression, biological activities and mechanisms of action of A20 (TNFAIP3). Biochem. Pharmacol. *80*, 2009–2020.

Vince, J.E., Wong, W.W.-L., Gentle, I., Lawlor, K.E., Allam, R., O'Reilly, L., Mason, K., Gross, O., Ma, S., Guarda, G., et al. (2012). Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity *36*, 215–227.

Wilkinson, K.D., Tashayev, V.L., O'Connor, L.B., Larsen, C.N., Kasperek, E., and Pickart, C.M. (1995). Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. Biochemistry *34*, 14535–14546.

Xia, Z.-P., Sun, L., Chen, X., Pineda, G., Jiang, X., Adhikari, A., Zeng, W., and Chen, Z.J. (2009). Direct activation of protein kinases by unanchored polyubiquitin chains. Nature *461*, 114–119. Immunity Supplemental Information

## A20 Restricts Ubiquitination

## of Pro-Interleukin-1ß Protein Complexes

### and Suppresses NLRP3 Inflammasome Activity

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#### SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. NFκ B-induced expression levels of pro-IL-1β and NLRP3 correlate poorly with spontaneous pro-IL-1β processing. (A) Control and A20-deficient BMDMs on  $Tnf^{-/-}$  background were stimulated with the indicated TLR ligands for 2 h or 6 h. The ligands used were 1 µg/mL Pam3CSK4 (P3CSK4; TLR2), 100 µg/mL poly(I:C) (P(I:C); TLR3), 500 ng/mL lipopolysaccharide (LPS; TLR4), 100 ng/mL flagellin (Fla; TLR5), 3 µg/mL gardiquimod (Gdq; TLR7), and 5 µM CpG (TLR9). Subsequently, cells were lysed and analyzed by immunoblotting for the indicated proteins. (B) *Wt*,  $Tnfaip3^{-/-}Myd88^{+/+}$ ,  $Tnfaip3^{+/+}Myd88^{-/-}$ , and  $Tnfaip3^{-/-}Myd88^{-/-}$  BMDMs were stimulated with 100 µg/mL poly(I:C) or 500 ng/mL LPS for 2 h or 6 h. Cells were subsequently lysed and immunoblotted for the indicated proteins. (C) *Wt*,  $Tnfaip3^{-/-}Ripk3^{+/+}$ ,  $Tnfaip3^{+/+}Ripk3^{-/-}$ , and  $Tnfaip3^{-/-}BMDMs$  stimulated with 500 ng/mL LPS for 10 to 120 minutes were analyzed by immunoblotting for the indicated proteins.

Figure S2, related to Figure 2. RIPK1 catalytic activity is required for spontaneous IL-1 $\beta$  processing in A20-deficient cells without formation of the ripoptosome. (A) A20-deficient BMDMs were stimulated for ~15 h with 500 ng/mL LPS in the presence or absence of 7.8-250  $\mu$ M Necrostatin-1 (diluted 2-fold serially). IL-1 $\beta$  secretion in each condition was analyzed by ELISA. (B) RIPK1 IPs were performed on lysates of control or A20-deficient BMDMs stimulated with 500 ng/mL LPS for 0-12 h. Co-association of each indicated protein with RIPK1 was subsequently analyzed by immunoblotting. Input

western analyses are shown on the right. To avoid potential complications by the contribution of TNF $\alpha$  signaling to RIPK1 functions, control and A20-deficient cells on  $Tnf^{-/-}$  background were used. Similar results have been obtained from cells on  $Tnf^{+/+}$  background. Arrows indicate cleaved RIPK1.

Figure S3, related to Figure 4. Pro-IL-1 $\beta$ -associated ubiquitination does not depend on NLRP3, ASC, or caspase-1. (A-C) BMDMs of each indicated genotype were stimulated with 500 ng/mL LPS with or without 5 mM ATP for the last 30 minutes. Pro-IL-1 $\beta$  IPs were then performed, followed by immunoblotting for Ub signals and the interaction of each indicated protein with pro-IL-1 $\beta$ .

Figure S4, related to Figure 1. A20-deficient cells do not exhibit increased AIM2 inflammasome activity following TLR2 and TLR7 prestimulation. BMDMs derived from *wt*,  $Tnfaip3^{-/-}Asc^{+/+}$ ,  $Tnfaip3^{+/+}Asc^{-/-}$ , and  $Tnfaip3^{-/-}Asc^{-/-}$  mice were pre-stimulated with 1 µg/mL Pam3CSK4, 3 µg/mL gardiquimod, or 500 ng/mL LPS for ~3 h. Subsequently, cells were transfected with 1 µg/mL poly(dA:dT) for ~6 h. Supernatants harvested from each condition were then analyzed for IL-1 $\beta$  secretion by ELISA (A) and LDH release (B). Each bar in graphs represents mean of triplicate wells + s.d. \* denotes statistical significance by student t test (p < 0.05). n.s. = not statistically significant.

#### SUPPLEMENTAL EXPERIMENTAL PROCEDURES

#### **BMDM** cultures and treatments

Primary BMDMs were cultured from mouse femurs and tibia using standard protocols (Zhang et al., 2008). Briefly, femurs and tibia from each mice were combined, crushed in mortar and pestle to release cells from the marrows, and grown in Complete DMEM (containing 10% FBS, pen-strep antibiotics, L-Glut, 55 µM 2-mercaptoethanol, nonessential amino acids, and sodium pyruvate) supplemented with 10% conditioned media harvested from the M-CSF producing cell line, CMG14-12 (Takeshita et al., 2000). Between days 5-8, cells were harvested and resuspended at 2-4 x  $10^6$  cells per mL of Complete DMEM without M-CSF. For ELISAs, cells were plated at 1-2 million cells in 0.5 mL per well in 24-well format and allowed to settle for 2-18 hours prior to receiving stimuli. Zymosan, high molecular weight poly(I:C), LPS, flagellin, Gardiquimod, CpG were purchased from InvivoGen and used at the concentrations stated in figure legends. Anti-CD40 antibody, clone FGK4.5, was produced by the UCSF antibody core facility. Recombinant murine TNFa and Necrostatin-1 were purchased from R&D Systems and Enzo Life Sciences, respectively. Depending on the experiments, supernatants were harvested between 2-18 h post-stimulation. To stimulate BMDMs with ATP, BMDMs were first primed with LPS (50-500 ng/mL) for 4-16 h. Media were then aspirated, followed by addition of 0.5 mL PBS containing 5 mM ATP (Sigma). After incubation at 37°C for 15-30 min, PBS containing ATP was removed, and pre-warmed complete media without stimuli were applied. Supernatants were then harvested after 2-3 h of incubation

at 37°C. To prevent  $K^+$  efflux in some assays, KCl was used in place of NaCl in PBS formulation. For IP and IB analyses, 20 to 40 million cells were seeded in 10 cm dishes. After various treatments, cells were lifted using PBS containing 5 mM EDTA and pelleted by centrifugation.

#### **Protein overexpression analyses**

293T cells were grown to ~70-80% confluence in DMEM containing 10% FBS, Pen-Strep, and L-Glut before transfection using Lipofectamine LTX without Plus Reagent (Invitrogen). The following full-length cDNA expression plasmids were either generated using commercially available vectors or purchased from the indicated vendors: Myc-DDK tagged ASC in pCMV6-Entry (Origene); N-terminally HA-tagged caspase-1 in pCMV-HA (Clonetech); N-terminally-StrepTag mouse caspase-1 in pCMV-Script (Stratagene); Full-length IL-1 $\beta$  in pIRES2-AcGFP (Clonetech); N-terminally Flag-tagged A20 in pCMV-Tag2 (Stratagene). 3x HA-tagged wild-type Ub, K63-only Ub, K48R Ub, and K63R Ub pcDNA3.1 constructs were kind gifts from Dr. Vishva Dixit (Genentech). Cells were typically transfected in 6-well format in 2 mL media with 150 ng/well of each vector, except for Ub constructs (which were used at 3 µg/well) and A20 (which varied from 0-50 ng/well).

#### Cytokine ELISAs and LDH release assays

IL-1β and IL-18 ELISAs were performed using kits purchased from BD Biosciences and eBioscience, respectively, per kit instructions. LDH release assays were performed using Cytotoxicity Detection Kit (LDH) purchased from Roche Life Science.

#### Immunoblotting and immunoprecipitation

Pelleted cells were lysed in 0.5-1 mL lysis buffer consisting of TBS, pH 7.2-7.4, supplemented with 1% NP-40, Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Bioscience), 10 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM N-ethylmaleimide (NEM). After 30-60 min of agitation at 4°C, lysates were centrifuged at  $\sim$ 20,000g for 15 min to pellet insoluble debris for removal. For straight IB analyses, 10 µl of each lysate was run in 4-12% NuPAGE gels (Invitrogen). For IL-16 IP, each lysate (~1-2 mg total protein) was incubated overnight at 4°C with 5 µg of biotinylated hamster monoclonal IgG1, clone B122 (Biolegend). As control for specificity, 5 µg of biotinylated hamster IgG1 isotype control, clone G235-2356 (BD Biosciences) was used in place of clone B122. On the next day, 25 µl of Neutravidin Agarose (Pierce) was added into each lysate. After 1 h of incubation at 4°C, agarose resins were centrifuged, washed 3 times in lysis buffer, and heated at 85°C for 10 min in 30-40 µL 2x NuPAGE Sample Buffer containing 100 mM DTT prior to SDS-PAGE. Immunoprecipitations of caspase-1 and RIPK1 were performed similarly, except for using rabbit antibodies against caspase-1 (M-20, Santa Cruz Biotechnology) and RIPK1 (clone D94C12, Cell Signaling Technology) followed by Protein-G Agarose (Pierce). In those experiments, specificity controls were performed using rabbit anti-HA polyclonal antibody (Y11, Santa Cruz Biotechnology) or rabbit isotype control antibody (clone DA1E, Cell Signaling Technology), respectively. 0.2 μm polyvinylidene fluoride (PVDF) membranes were used for all western blotting analyses.

The following antibodies were used in IB analyses: rabbit anti-A20 (D13H3, Cell Signaling Technology); goat anti-IL-1ß (R&D Systems); rabbit anti-caspase-1, p10 (M-20, Santa Cruz Biotechnology); goat anti-caspase-1, p20 (G-19, Santa Cruz Biotechnology); rabbit anti-caspase-8 (clone D35G2, Cell Signaling Technology); rabbit anti-cleaved caspase-8, Asp387 (clone D5B2, Cell Signaling Technology); rabbit anti-RIPK1 (clone D94C12, Cell Signaling Technology); rabbit anti-RIPK3 (ProSci); mouse anti-Ub (clone P4D1, Santa Cruz Biotechnology); mouse anti-GAPDH (clone 6C5, Millipore); rabbit anti-HA (Y11, Santa Cruz Biotechnology); rabbit anti-Myc (M14, Santa Cruz Biotechnology); mouse anti-Flag (clone M2, Sigma-Aldrich). HRPconjugated secondary polyclonal antibodies to mouse, rabbit, and rat IgGs were purchased from Cell Signaling Technology; HRP-conjugated donkey polyclonal antibodies against goat IgGs were purchased from Jackson ImmunoResearch. Signals were developed using SuperSignal West Pico, Dura, or Femto Chemiluminescent Substrate (Pierce). To strip blots for reprobing, membranes were incubated in Restore Western Blot Stripping Buffer (Pierce) with gentle rocking motion for 20-30 minutes.

#### **De-ubiquitinase assays**

Five 10-cm plates of *Tnfaip3<sup>-/-</sup>* cells (~40 million cells per plate) were stimulated with 500 ng/mL LPS for 6 h. Cells were lysed, and IL-1 $\beta$  IPs were performed in 5 separate tubes as described in Supplemental Experimental Procedures. After the last wash, beads containing IL-1 $\beta$ -associated Ub complex were combined and washed once in DUB reaction buffer consisting of 50 mM Tris, 150 mM NaCl, 25 mM KCl, 5 mM MgCl<sub>2</sub>, and 1 mM DTT, pH7.2. Before centrifugation to pellet the beads, the reaction was split into

several aliquots for the different DUB treatments. Supernatants were then removed from the pelleted beads, and 20  $\mu$ L DUB reaction buffer was added. AMSH (a JAMM/MPN+ family of K63-specific DUB) (Cooper et al., 2009; McCullough et al., 2004), CylD (K63and linear Ub-specfic DUB) (Komander et al., 2008; 2009), isopeptidase-T (unanchored poly-Ub DUB) (Amerik AYu et al., 1997; Lindsey et al., 1998; Wilkinson et al., 1995), and USP2 (nonspecific DUB) (Baek et al., 1997) were all purchased from Boston Biochem. (For systematic comparisons of the specificity of each DUB, please see this recent paper (Mevissen et al., 2013)). 1  $\mu$ L of each DUB enzyme at the supplied concentration was added to each reaction. After incubating for 30-45 min at 37°C, each reaction was stopped by adding 20  $\mu$ L 4x NuPAGE Sample Buffer (Invitrogen) plus 5  $\mu$ L 1 M DTT, mixed, and heated at 85°C for 5-10 minutes prior to SDS-PAGE followed by immunoblotting as described above.

## Reversed-phase liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) analysis

*In-solution digestion.* Cell pellets containing  $\sim 8 \ge 10^8$  cells (corresponding to 20 mg of protein) were resuspended in 2 mL 8M urea plus 150 mM ammonium bicarbonate in the presence of 10 mM TCEP. Samples were then vortexed for 10 min, microtip sonicated at 30% with 3x 20 s pulses, and incubated at 56°C for 20 min. Subsequently, samples were alkylated by incubation with 20 mM iodoacetic acid for 45 min in the dark at room temperature, diluted to a final concentration of 2 M urea, and digested overnight at 37°C using TPCK modified trypsin (Thermo-Pierce) at an enzyme to substrate ratio of 2%. After digestion, samples were added with 4% formic acid, further diluted by adding 10

mL 0.1% formic acid, and subjected to peptide extraction using C18 SepPaks (Waters) according to the manufacturer's protocol. Eluates of the SepPaks were vacuum-evaporated.

*Enrichment of ubiquitinated peptides.* K- $\epsilon$ -GG enrichment was performed with the PTMScan® Ubiquitin Remnant Motif (K-e-GG) kit (Cell Signaling technology®), according to manufacturer's protocol. Peptides were resuspended in 1.4 mL immunoprecipitation buffer (IAP), bath sonicated, and centrifugated for 5 min at 1800 g. The supernatant was then transferred to microfuge tube containing 40  $\mu$ L of immunoaffinity beads and incubated for 2 hours at 4°C with rotation. Subsequently, the supernatant was removed by centrifugation at 2700 g for 1 min. Beads were washed twice with 1 mL IAP per wash, and then four times with 1 mL water per wash. After completely removing the supernatant in the last wash, peptides were recovered by adding 55  $\mu$ L 0.15% TFA to the beads, followed by a 10-min incubation. Supernatant was recovered, and the beads were washed with a second aliquot of 45  $\mu$ L 0.15% TFA. This supernatant was also recovered, combined with the previous aliquots, and evaporated to dryness.

*Reverse-phase LC-MS/MS Analysis.* Peptides were resuspended in 10  $\mu$ L 0.1% formic acid, and analyzed by nanoflow-UPLC-HCD-MSMS. Samples were then separated by nano-flow liquid chromatography using a 75  $\mu$ m x 150 mm reverse phase 1.7  $\mu$ m BEH 130 C18 column (Waters) at a flow rate of 600 nL/min in a NanoAcquity UPLC system (Waters). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Following equilibration of the column in 2% solvent B, an aliquot of each digest (5  $\mu$ L) was injected; then the organic content of the mobile phase

was increased linearly to 30% over 99 min, and then to 50% in 2 min. The liquid chromatography eluate was coupled to a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray ion source. Spraying was from an uncoated 15 µm inner diameter spraying needle (New Objective, Woburn, MA). Peptides were analyzed in positive ion mode and in information-dependent acquisition mode to automatically switch between MS and MS/MS acquisition. MS spectra were acquired in profile mode using the Orbitrap analyzer in the m/z range between 350 and 1500. For each MS spectrum, the 6 most intense multiple charged ions over a threshold of 2000 counts were selected to perform HCD experiments. Product ions were analyzed on the Orbitrap in centroid mode. HCD activation time was set to 100 ms; normalized collision energy was set to 30. A dynamic exclusion window of 0.5 Da was applied that prevented the same m/z from being selected for 60 s after its acquisition.

Peak lists were generated using PAVA in-house software (Guan et al., 2011), based on the RawExtract script from Xcalibur v2.4 (Thermo Fisher Scientific, San Jose, CA). The peak lists were searched against the murine subset of the UniProtKB database as of March 21, 2012, containing 77771 entries, using in-house ProteinProspector version 5.8.0 (a public version is available online) (Clauser et al., 1999). A randomized version of all entries was concatenated to the database for estimation of false discovery rates in the searches. Peptide tolerance in searches was 20 ppm for precursor and 20 ppm for product ions. respectively. Peptides containing two miscleavages allowed. were Carboxymethylation of cysteine was allowed as constant modification; acetylation of the N terminus of the protein, pyroglutamate formation from N terminal glutamine, oxidation

of methionine and GlyGly addition on lysines or the N terminal amino, were allowed as variable modifications. The number of modification was limited to two per peptide. A minimal ProteinProspector protein score of 20, a peptide score of 15, a maximum expectation value of 0.001 for protein and 0.1 for peptide and a minimal discriminant score threshold of 0.0 were used for identification criteria.

#### REFERENCES

Amerik AYu, Swaminathan, S., Krantz, B.A., Wilkinson, K.D., and Hochstrasser, M. (1997). In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. The EMBO Journal *16*, 4826–4838.

Baek, S.H., Choi, K.S., Yoo, Y.J., Cho, J.M., Baker, R.T., Tanaka, K., and Chung, C.H. (1997). Molecular cloning of a novel ubiquitin-specific protease, UBP41, with isopeptidase activity in chick skeletal muscle. The Journal of Biological Chemistry *272*, 25560–25565.

Clauser, K.R., Baker, P., and Burlingame, A.L. (1999). Role of accurate mass measurement (+/- 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Analytical Chemistry *71*, 2871–2882.

Cooper, E.M., Cutcliffe, C., Kristiansen, T.Z., Pandey, A., Pickart, C.M., and Cohen, R.E. (2009). K63-specific deubiquitination by two JAMM/MPN+ complexes: BRISC-associated Brcc36 and proteasomal Poh1. The EMBO Journal *28*, 621–631.

Guan, S., Price, J.C., Prusiner, S.B., Ghaemmaghami, S., and Burlingame, A.L. (2011). A data processing pipeline for mammalian proteome dynamics studies using stable isotope metabolic labeling. Molecular & Cellular Proteomics : MCP *10*, M111.010728.

Komander, D., Lord, C.J., Scheel, H., Swift, S., Hofmann, K., Ashworth, A., and Barford, D. (2008). The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module. Molecular Cell *29*, 451–464.

Komander, D., Reyes-Turcu, F., Licchesi, J.D.F., Odenwaelder, P., Wilkinson, K.D., and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Reports *10*, 466–473.

Lindsey, D.F., Amerik, A., Deery, W.J., Bishop, J.D., Hochstrasser, M., and Gomer, R.H. (1998). A deubiquitinating enzyme that disassembles free polyubiquitin chains is required for development but not growth in Dictyostelium. The Journal of Biological Chemistry *273*, 29178–29187.

McCullough, J., Clague, M.J., and Urbé, S. (2004). AMSH is an endosome-associated ubiquitin isopeptidase. The Journal of Cell Biology *166*, 487–492.

Mevissen, T.E.T., Hospenthal, M.K., Geurink, P.P., Elliott, P.R., Akutsu, M., Arnaudo, N., Ekkebus, R., Kulathu, Y., Wauer, T., Oualid, El, F., et al. (2013). OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. Cell *154*, 169–184.

Takeshita, S., Kaji, K., and Kudo, A. (2000). Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J. Bone Miner. Res. *15*, 1477–1488.

Wilkinson, K.D., Tashayev, V.L., O'Connor, L.B., Larsen, C.N., Kasperek, E., and Pickart, C.M. (1995). Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. Biochemistry *34*, 14535–14546.

Zhang, X., Goncalves, R., and Mosser, D.M. (2008). The isolation and characterization of murine macrophages. Current Protocols in Immunology / Edited by John E. Coligan ... [Et Al.] *Chapter 14*, Unit14.1.



#### Figure S2.







