SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. NFκB-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*$^{-/-}$*Ripk3*$^{-/-}$ 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-1β 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 μM Necrostatin-1 (diluted 2-fold serially). IL-1β 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 TNFα 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-1β-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-1β IPs were then performed, followed by immunoblotting for Ub signals and the interaction of each indicated protein with pro-IL-1β.

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-1β 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, non-essential 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 TNFα 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-1β 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_3VO_4, and 10 mM N-ethylmaleimide (NEM). After 30-60 min of agitation at 4°C, lysates were centrifuged at ~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-1β 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-1β (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). HRP-conjugated 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*$^{-/-}$ cells (~40 million cells per plate) were stimulated with 500 ng/mL LPS for 6 h. Cells were lysed, and IL-1β IPs were performed in 5 separate tubes as described in Supplemental Experimental Procedures. After the last wash, beads containing IL-1β-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 $MgCl_2$, 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 μL DUB reaction buffer was added. AMSH (a JAMM/MPN+ family of K63-specific DUB) (Cooper et al., 2009; McCullough et al., 2004), CylD (K63- and 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 μL 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 μL 4x NuPAGE Sample Buffer (Invitrogen) plus 5 μL 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 ~8 x 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 37ºC 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-ε-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 μL 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 μL 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 μL 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 μL 0.1% formic acid, and analyzed by nanoflow-UPLC-HCD-MSMS. Samples were then separated by nano-flow liquid chromatography using a 75 μm x 150 mm reverse phase 1.7 μm 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 μL) 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 were allowed. 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.

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Figure S1. **Duong, et al.**

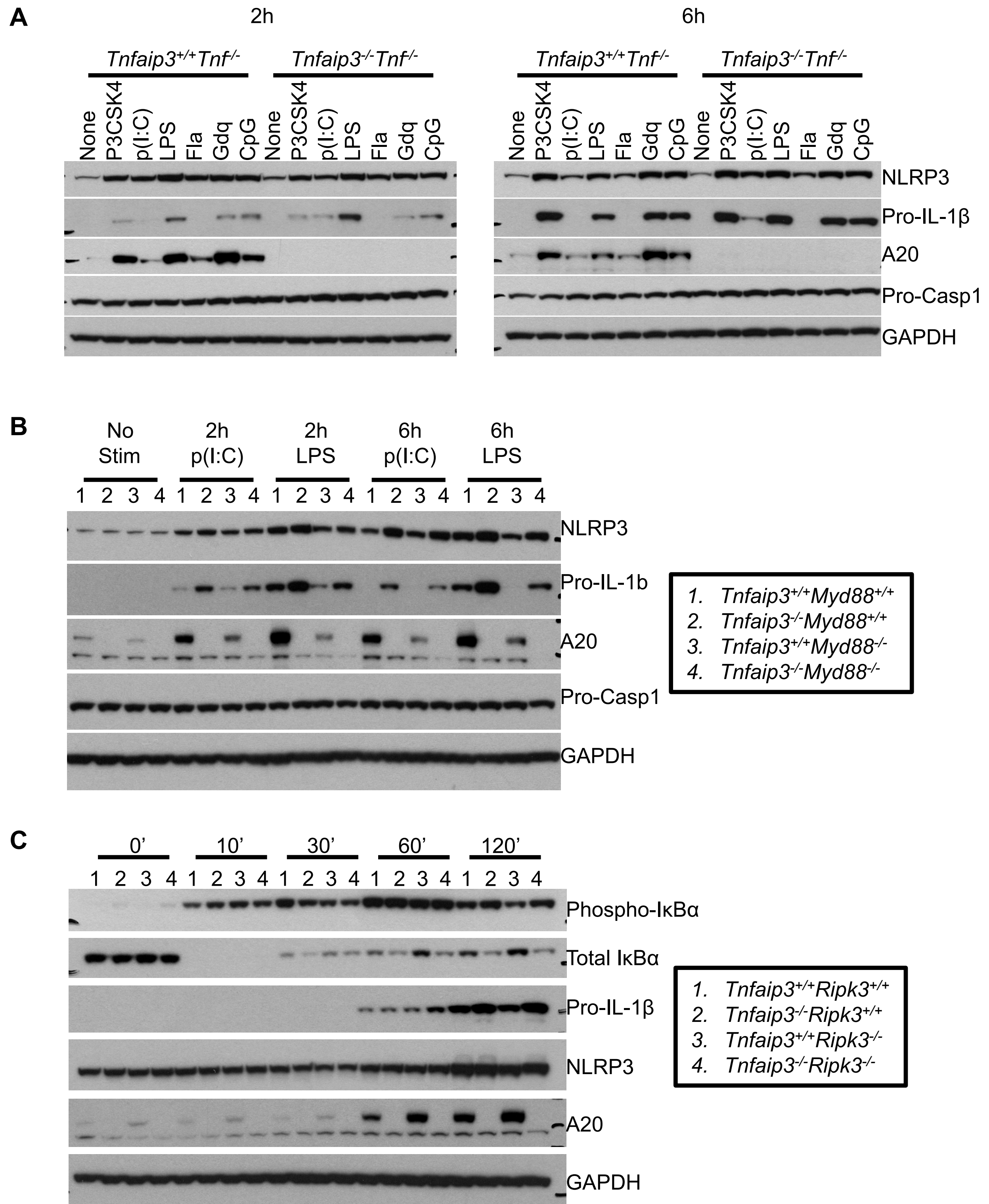
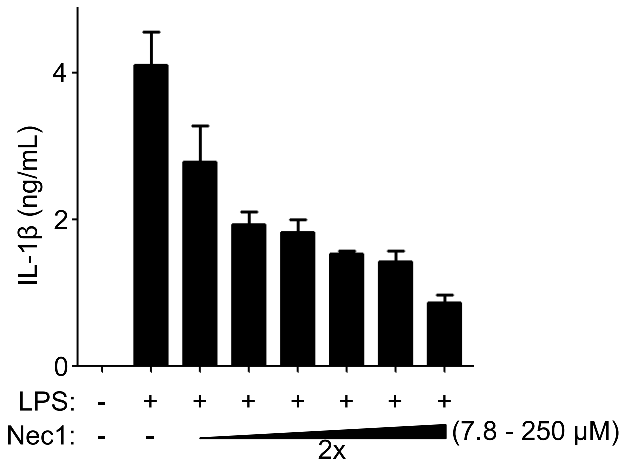


Figure S2. **Duong, et al.**

A



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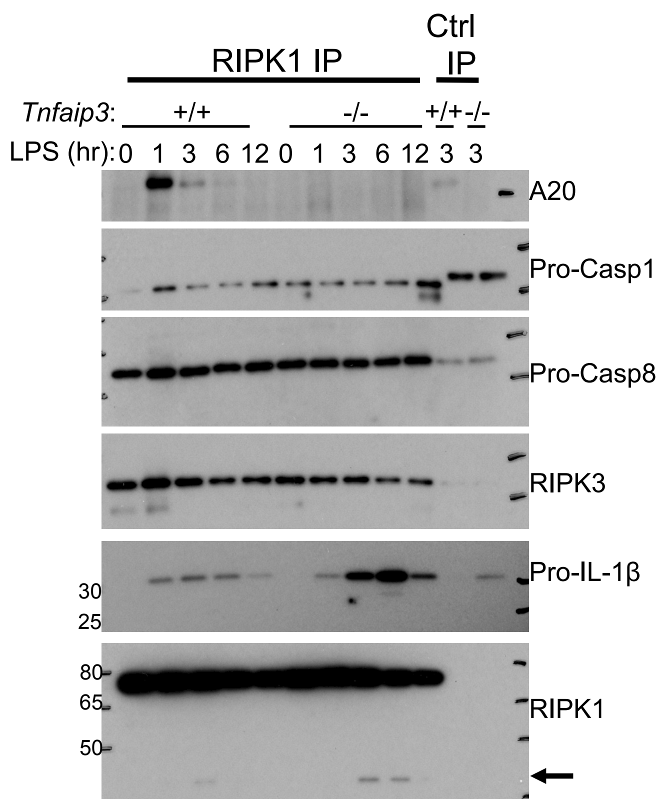

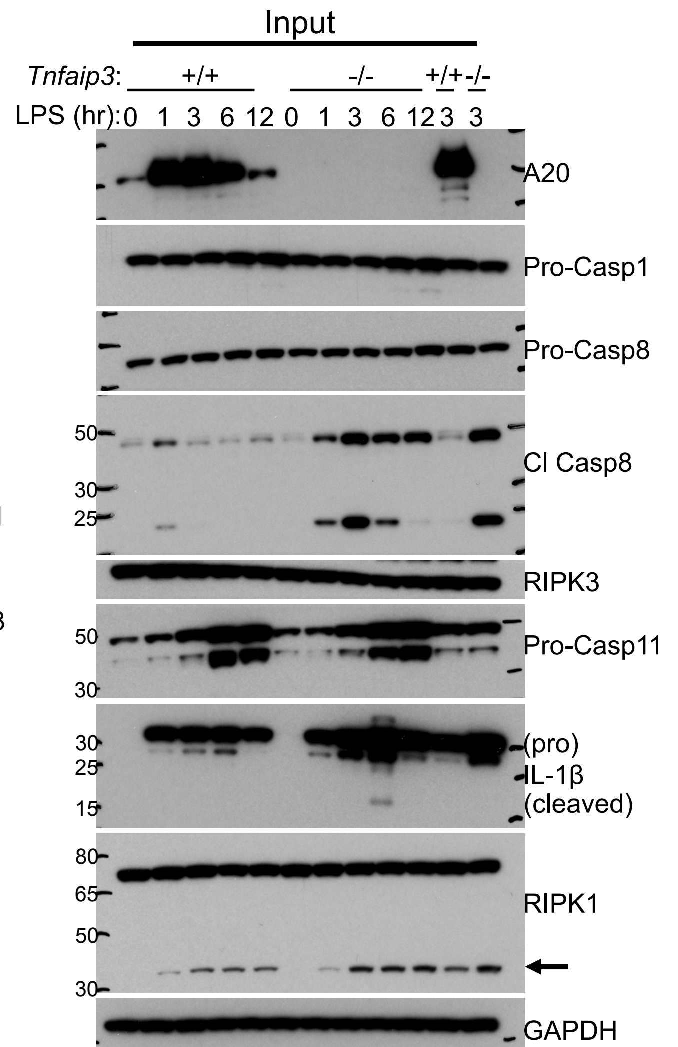

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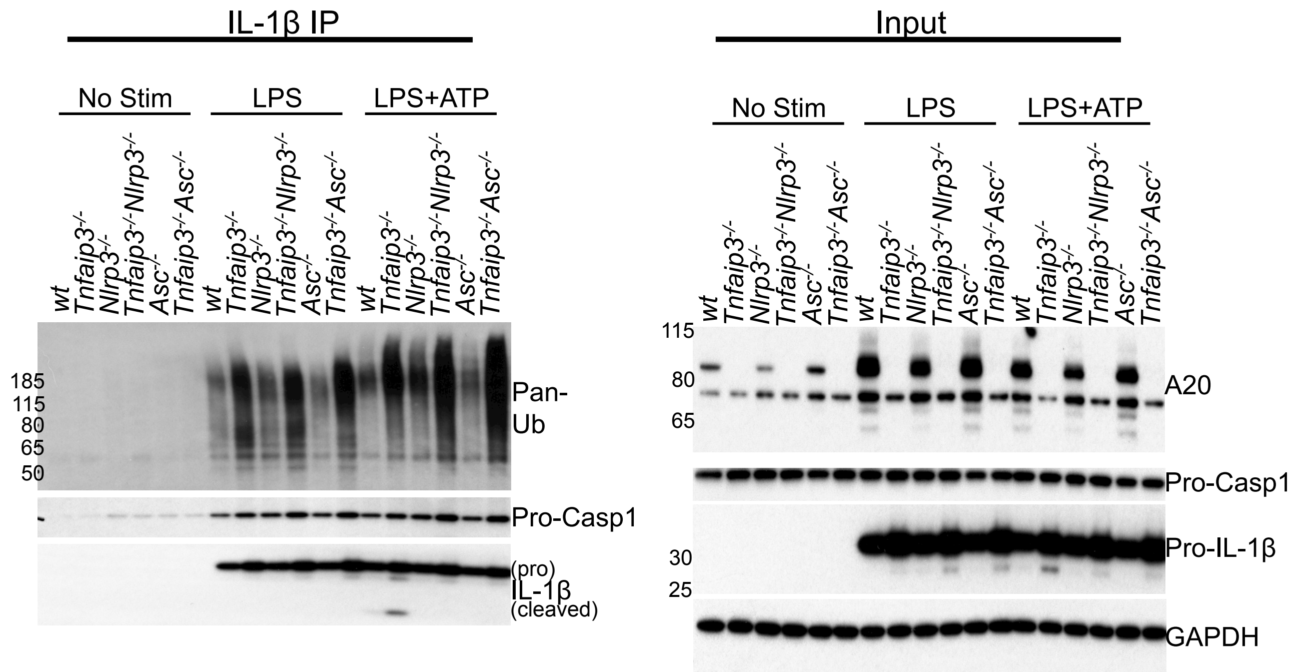

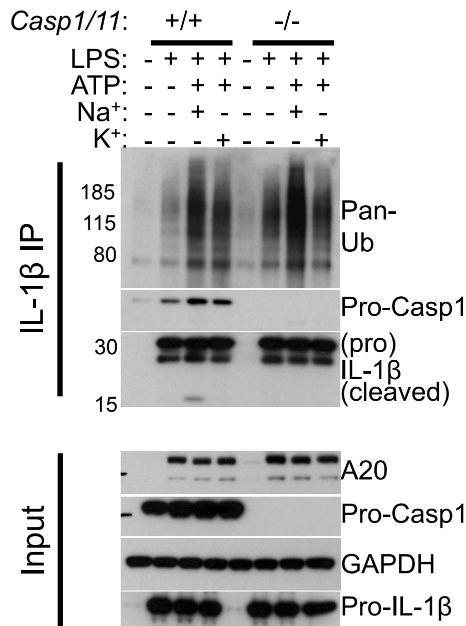

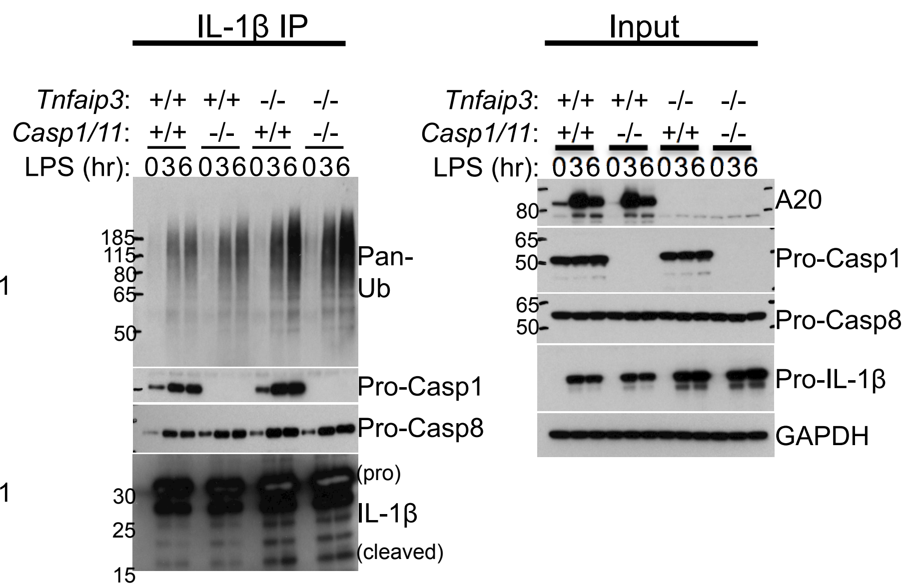

Figure S4. **Duong, et al.**