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Deubiquitinases Regulate the Activity of Caspase-1 and Interleukin-1β Secretion via Assembly of the Inflammasome*[S]

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Background: The inflammasome is a multimolecular complex that regulates the processing of the pro-inflammatory cytokine interleukin-1β.
Results: Inhibitors of deubiquitinase (DUB) enzymes inhibited the release of interleukin-1β.
Conclusion: DUBs regulate assembly of the inflammasome.
Significance: DUBs may represent new anti-inflammatory drug targets for the treatment of inflammatory disease.

IL-1β is a potent pro-inflammatory cytokine produced in response to infection or injury. It is synthesized as an inactive precursor that is activated by the protease caspase-1 within a cytosolic molecular complex called the inflammasome. Assembly of this complex is triggered by a range of structurally diverse damage or pathogen associated stimuli, and the signaling pathways through which these act are poorly understood. Ubiquitination is a post-translational modification essential for maintaining cellular homeostasis. It can be reversed by deubiquitinase enzymes (DUBs) that remove ubiquitin moieties from the protein thus modifying its fate. DUBs present specificity toward different ubiquitin chain topologies and are crucial for recycling ubiquitin molecules before protein degradation as well as regulating key cellular processes such as protein trafficking, gene transcription, and signaling. We report here that small molecule inhibitors of DUB activity inhibit inflammasome activation. Inhibition of DUBs blocked the processing and release of IL-1β in both mouse and human macrophages. DUB activity was necessary for inflammasome association as DUB inhibition also impaired ASC oligomerization and caspase-1 activation without directly blocking caspase-1 activity. These data reveal the requirement for DUB activity in a key reaction of the innate immune response and highlight the therapeutic potential of DUB inhibitors for chronic auto-inflammatory diseases.

Inflammation is a response of the innate immune system to infection or injury. During inflammation, signaling cascades are triggered that result in the synthesis of pro-inflammatory mediators, including members of the interleukin-1 (IL-1) cytokine family, such as IL-1β and IL-1α (1). IL-1β is a potent pro-inflammatory molecule, and its overproduction is associated with chronic inflammation and the development of pathologies such as gout, diabetes, or cancer (2). IL-1β production is, therefore, tightly regulated at transcriptional and post-transcriptional levels (3). IL-1β is synthesized as an inactive precursor molecule (pro-IL-1β, 31 kDa) by cells of the innate immune system such as macrophages upon the activation of pattern recognition receptors by pathogen- or damage-associated molecular patterns (1). Activated macrophages then require an additional stimulus to trigger the assembly of a multimolecular complex called the inflammasome that results in the activation of caspase-1 and the processing of pro-IL-1β to an active, secreted IL-1β molecule (4). Inflammasomes are typically formed by a cytosolic pattern recognition receptor of the Nod-like receptor (NLR)[2] family, such as NLRP1, NLRP3, NLRP6 or NLRC4 (5), or members of the PYHIN family such as AIM-2 (absent in melanoma 2) receptor (6). The assembly of both NLRP3 and AIM2 inflammasomes requires the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) to bring the receptor and the zymogen pro-caspase-1 into close proximity (4, 6). This results in the activation of caspase-1 and processing and secretion of IL-1β (7). The specificity of inflammasome signaling depends upon the pathogen-associated molecular pattern or the damage-associated molecular pattern that the cell encounters. For example, NLRP3 is reported to sense a range of structurally diverse pathogen- and damage-associated molecular patterns (4), whereas AIM2 directly binds double-stranded DNA (8), and NLRC4 appears to detect the

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[2] The abbreviations used are: NLR, Nod-like receptor; DUB, deubiquitinase; USP, ubiquitin-specific protease; UCH, ubiquitin C-terminal hydrolase; MSU, mono-sodium urate; LC3, light chain 3; (c)IAP, (cellular) inhibitor of apoptosis; ASC, apoptosis-associated speck-like protein containing a CARD; ESI, eeyarestatin I; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Z-, benzyloxycarbonyl; AFC, aminofluoromethylcoumarin; ER, endoplasmic reticulum.

presence of intracellular pathogens by sensing flagellin (9). However, the endogenous signaling mechanisms that coordinate inflammasome formation are largely unknown.

Post-translational protein modifications, such as phosphorylation or ubiquitination, provide the means by which cells integrate the presence and meaning of extracellular signals (10). Ubiquitination involves the attachment of ubiquitin, a process regulated by three enzymes, E1, E2, and E3. E1 is an ubiquitin-activating enzyme and mediates the formation of an E1-ubiquitin thiol ester bond. E2, a ubiquitin-conjugating enzyme, mediates the transfer of ubiquitin from E1 to the target protein and interacts with E3, a ubiquitin-protein ligase, that finally conjugates ubiquitin to a lysine of the target protein (11). The functional outcome of this modification depends upon the nature of ubiquitination and the specific linkage between ubiquitin molecules (10). Ubiquitination can be reversed by deubiquitinases (DUBs), enzymes that remove ubiquitin from proteins. There are ~90 different predicted DUBs in humans that can selectively recognize different types of ubiquitin chains (12). These can be grouped into five different families; the ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumor proteases, Machado-Josephin domains, and the JAB1/MPN/MOV34 metalloenzymes (12). DUBs are often associated with ubiquitin recycling and protein degradation by the proteasome (11). However, DUBs are also known to promote protein stability, to directly regulate protein activity, and to form protein scaffolds independent of their proteolytic activity. Indeed, several DUBs have been shown to play a crucial role in ubiquitin-mediated cellular processes such as membrane trafficking, signaling, and gene transcription (12, 13).

Post-translational modifications are important for inflammasome formation. Phosphorylation/dephosphorylation is required because the protein phosphatase inhibitors okadaic acid and calyculin A are reported to inhibit the formation of a number of inflammasomes (14). Phosphorylation of NLRC4 is also required for NLRC4 inflammasome activation by *Salmonella typhimurium* infection (15). There is increasing evidence that ubiquitination is also important for the regulation of IL-1β processing and release. The cellular inhibitors of apoptosis, cIAP1 and cIAP2, have ubiquitin ligase activity and modulate the release of IL-1β in response to the activation of multiple inflammasomes by direct ubiquitination of caspase-1 (16). Depletion of IAP proteins can also activate a RIP3 kinase-dependent IL-1β processing and secretion that is partially dependent upon caspase-8 (16, 17). The ubiquitin ligase-associated protein SGT1 also interacts with the NLRP3 inflammasome and regulates its activity (18). Recent findings have shown that DUBs are required for assembly of the NLRP3 inflammasome (19). The objective of the study presented here was also to investigate the role of DUBs in inflammasome activation and IL-1β secretion.

Here, we provide evidence that in both murine and human macrophages DUBs regulate the release of IL-1β and that this process is independent of proteasome activity or protein translocation. In addition, our data suggest that DUB inhibitors act upstream of caspase-1 and are required for ASC oligomerization and speck formation. These data validate recent findings regarding the role of DUBs in this process (19) and, crucially, provide further insights into their mechanisms of regulation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Bacterial lipopolysaccharide (LPS, *Escherichia coli* 026:B6) was purchased from Sigma. Fetal bovine serum (FBS) was obtained from PAA Laboratories. The primary antibodies used for the Western blot were anti-IL-1β and IL-1α (anti-mouse and anti-human) from R&D, rabbit anti-ASC (sc-22514-R, Santa Cruz), anti UCH37 (EP4897, Novus Biologicals), anti-LC3 (M115–3, Medical and Biology Laboratories), ubiquitin (sc-8017, Santa Cruz), anti-caspase-1 p10 (sc-515, Santa Cruz), and anti-β-actin-HRP (Sigma A3854). Secondary antibody HRP conjugates used for Western blot were from DAKO (1:1000). Alexa Fluor 594-conjugated donkey anti-rabbit antibody (A-21207, Invitrogen) was used for immunohistochemistry. Lipofectamine2000 was from Invitrogen. Eeyarestatin I (ESI) and ESR35 were synthesized at the University of Manchester as previously published (20), cpd A was from Novartis, b-AP15 was donated by Prof. Stig Linder, WP1130 was from Selleck, IU1 was from Calbiochem, MG-262 was from Calbiochem, and bortezomib was from Selleck.

Cells and Treatments—Macrophages were prepared from adult male C57BL/6 mice (Harlan) as described previously (21). Briefly, the peritoneal cavity was gently lavaged with RPMI 1640 media (Sigma). Cells were collected by centrifugation of the recovered media (250 × *g*, 5 min) and plated in 24-well plates at a density of 5×10^5 cells/well in RPMI 1640 media (Sigma) supplemented with 5% FBS (PAA Laboratories), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma). The macrophages were allowed to adhere overnight (37 °C, 5% CO_2) and washed with fresh medium to remove unattached cells before use. Cultured peritoneal macrophages were LPS-primed (1 μg/ml, 2h) to induce pro-IL-1β expression before ATP treatment.

THP-1 cells were cultured in RPMI 1640 media (Sigma) supplemented with 10% FBS (PAA Laboratories), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma). Cells were plated in 24-well plates at a density of 5×10^5 cells/well and treated with phorbol 12-myristate 13-acetate (0.5 μM). After 3 h, the medium was removed, fresh media was added, and cells were incubated overnight (37 °C, 5% CO_2). Cells were then LPS-primed (1 μg/ml, 4 h) to induce pro-IL-1β expression before nigericin treatment.

Cells were treated for 15 min with vehicle (0.5% DMSO) or the indicated inhibitors ESI (50 μM unless otherwise stated), ESR35 (50 μM), ES24 (50 μM), ESI-B (50 μM), cpd A (10 μM), b-AP15 (1 μM), WP1130 (1 μM), IU1 (50 μM), MG-262 (20 μM), or bortezomib (50 nM) before the addition of ATP (5 mM, 20 min), nigericin (10 μM, 60 min), or mono-sodium urate (MSU; 250 μg/ml, 60 min). For the AIM2 activating experiments, endotoxin-free plasmid DNA (empty mammalian expression vector pCMV-Tag4B) was prepared using a Qiagen EndoFree plasmid MAXI kit. After LPS treatment, macrophages were transferred to serum and antibiotic free media and treated with inhibitors immediately before transfection with pCMV-Tag4B (1.6 μg/ml) using Lipofectamine (4 μl per 1 ml of media) according to the manufacturer's instructions. Controls included DNA or

Lipofectamine treatment alone. Cells were incubated for 4 h before the collection of supernatants and cell lysates for analysis of IL-1 release.

ELISA—Macrophage supernatants were analyzed for IL-1α and IL-1β using specific ELISA kits from R&D Systems according to the manufacturer's instructions.

Western Blots—Cells were collected in lysis buffer (HEPES 50 mM, EGTA 0.1 mM, EDTA 0.1 mM, NaCl 120 mM, Nonidet P-40 0.5%, pH 7.5, protease inhibitors), and the insoluble fraction was removed by centrifugation (12500 × *g*, 10 min). Supernatants and cell lysates (10–15 μg) were resolved on 15% polyacrylamide gels for detection of IL-1β, IL-1α, UCH37, and light chain 3 (LC3) and 10% gels for detection of ubiquitinated proteins. Proteins were transferred to a nitrocellulose membrane, and specific proteins were detected by Western blotting with anti-IL-1β (1:1000), anti-ubiquitin (1:1000), anti-LC3 (1:1000), or anti-UCH37 (1:1000) followed by a secondary HRP-conjugated antibody or with anti-β-actin-HRP and subsequently detected using enhanced chemiluminescence reagents (ECL, Amersham Biosciences).

ASC Speck Detection and Quantification—Cells were plated as described above on glass coverslips. Peritoneal macrophages or THP-1 cells were LPS-primed (1 μg/ml, 2 or 4 h, respectively). After b-AP15 or DMSO 0.5% treatment, cells were activated with ATP or nigericin and fixed with 4% paraformaldehyde, 4% sucrose in PBS for 30 min. The cells were permeabilized with 0.1% Triton X-100 and then quenched with 0.25% ammonium chloride. A blocking step for 1 h using 5% BSA, 5% donkey serum (block solution) was used before incubation with the rabbit anti-ASC (1:50) in block solution for 1 h. Coverslips were then washed in PBS. ASC antibodies were detected by incubation with Alexa Fluor 594 conjugated donkey anti-rabbit antibody (1:200) in blocking solution for 1 h. The coverslips were washed again with PBS and finally in distilled water before being dried and mounted onto a glass slide using ProLong Gold mounting medium containing DAPI (Invitrogen). Images were taken with an Olympus BX51 upright microscope using a 10×/0.50 Plan Fln objective and captured using a Coolsnap EZ camera (Photometrics) through MetaVue Software (Molecular Devices). To quantify the extent of speck formation, the percentage of cells that contained an ASC speck was counted. Cells from 10 different fields (average of 200 cells/field for peritoneal macrophages and 650 cells/field for THP-1) were counted for each of the different experiments ($n = 3$ for THP-1 and $n = 2$ for peritoneals). Images were analyzed using ImageJ (rsb.info.nih.gov). The data are expressed as the percentage of ASC specks per number of cells per field.

ASC Oligomerization Assay—Cross-linking of ASC oligomers was performed as previously described (22). Briefly, cells were lysed in buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 320 mM sucrose, Halt Protease and Phosphatase Inhibitor Mixture (Thermo Fisher)) by syringing 40× on ice using a 1-ml syringe with a 25-gauge needle. The cell lysate was centrifuged at 2000 rpm, and the supernatant (cell lysate) was diluted with one volume of CHAPS buffer (20 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.1% CHAPS, and Halt Protease and Phosphatase Inhibitor Mixture) and then centrifuged at 5000 rpm to pellet the ASC oligomers. This final step was repeated twice. ASC oligomers were cross-linked using 2 mM NHS-Diazirine (SDA) cross-linkers (Thermo Fisher) for 30 min at 4 °C in CHAPS buffer. UV irradiation (using a 365-nm UV bulb) was performed for 15 min at room temperature before adding lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). Proteins were then separated on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membrane (0.2 μm pore size). Specific proteins were detected by Western blotting as described in the Western blot section using anti-IL-1β (1:1000), anti-ASC (1:1000), or anti-caspase-1 (1:300) antibodies.

Silencing of UCH37 with siRNA—Accell SMARTpool siRNA for human UCH37 or control was purchased from Thermo Scientific Dharmacon, and the manufacturer's protocol was followed for the delivery of siRNAs into the cells. Briefly a suspension of THP-1 cells was prepared in Accell delivery media (Dharmacon). siRNA, control or UCH37, was added to the cell suspension to a final concentration of 1 μM. The cells were incubated for 72h (37 °C, 5% CO_2). Phorbol 12-myristate 13-acetate was added to each well to a final concentration of 0.1 μM, and cells incubated overnight (37 °C, 5% CO_2). The following day cells were primed with LPS (1 μg/ml, 4 h) before nigericin was added as previously described. Where stated, cells were incubated with IU-1 (50 μM) before nigericin treatment.

Analysis of Fibroblast Growth FGF Factor-1 (FGF1), FGF2, and IL-1α Release—NIH3T3 cells were used to study the effect of ESI upon the non-conventional release of proteins 48 h after adenoviral transduction with FGF1:HA, FGF2:HA, or mature IL-1α:V5. A heat shock-induced release assay was performed by incubation of the cells at 37 °C or 42 °C for 110 min in serum-free DMEM containing 5 units/ml of heparin (Sigma) in the presence or absence of 25 μM ESI or ESR35. Cell viability was assessed by measuring lactate dehydrogenase activity in the medium using the Promega CytoTox kit. The heat shock treatment did not cause significant NIH3T3 cell death. To assess the export of FGF1, conditioned media were collected and briefly centrifuged at 1000 × *g* to remove detached cells, and FGF1 was isolated for immunoblot analysis using heparin-Sepharose chromatography as described (23). FGF1 was eluted from heparin-Sepharose with 3 M NaCl, concentrated using Amicon concentrator filter units (cutoff 10 kDa), resolved by SDS-PAGE, and Western-blotted using rabbit anti-FGF1 antibodies produced in the Prudovsky laboratory. 2% of the cell lysate and 50% of FGF1 isolated from the medium were loaded per SDS-PAGE lane. FGF2 has a stronger affinity to heparan sulfate proteoglycans of the cell surface than FGF1. For this reason, after collecting the medium cells were washed with a small volume (10% of medium volume) of the following buffer: 2 M NaCl in 10 mM Tris-HCl, pH 7.2, supplemented with 0.5% BSA. The high salt wash buffer was then mixed with the previously collected medium. Isolation and detection of FGF2 was performed similarly to FGF1 except that a commercial (Covance) mouse monoclonal anti-HA antibody was used for Western blotting. IL-1α has a very low affinity to heparin. Thus, conditioned media were directly concentrated using Amicon centrifugal devices (cutoff 10 kDa), resolved by SDS-PAGE, and West-

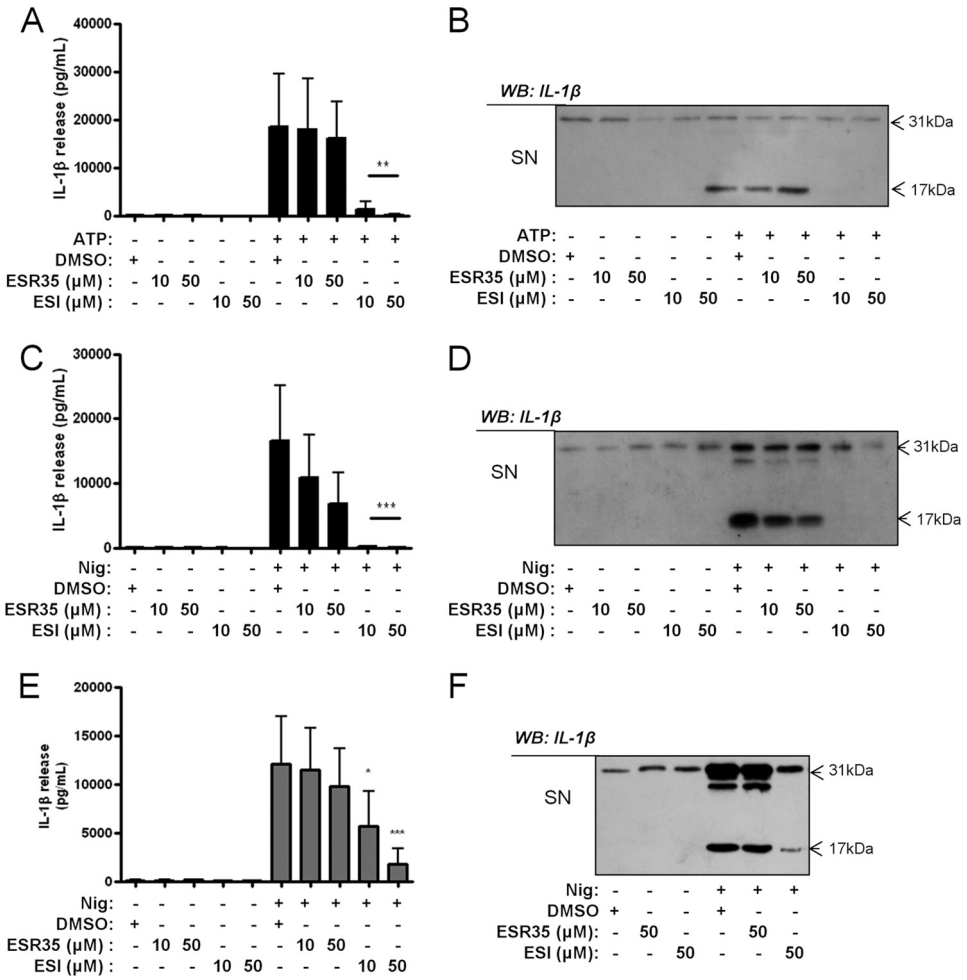

FIGURE 1. **Eeyarestatin I blocks the release of IL-1β from macrophages.** LPS-primed (1 μg/ml, 2 h) peritoneal macrophages (*black columns*) were incubated with DMSO (0.5%), ESI, or ESR35 (10 and 50 μM) for 15 min before a 20-min incubation with 5 mM ATP (*A* and *B*) or 10 μM nigericin (*Nig*, *C* and *D*). LPS-primed (1 μg/ml, 4 h) THP-1 cells (*gray columns*) were incubated with DMSO (0.5%), ESI, or ESR35 (10 and 50 μM) before a 1-h incubation with 10 μM nigericin (*E* and *F*). Supernatants (*SN*) were collected and analyzed for IL-1β by ELISA (*A*, *C*, and *E*) and Western blot (*WB*; *B*, *D*, and *F*). In the blots (*B*, *D*, and *F*) the band at 31 kDa represents pro-IL-1β, and the band at 17 kDa represents mature IL-1β. Data are presented as the mean of 4 separate experiments ± S.D. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. All inhibitor groups were compared with ATP or nigericin alone.

ern-blotted using a mouse monoclonal anti-V5 antibody (Invitrogen).

Caspase-1 Activity Assays—The caspase-1 activity of THP-1 cells was determined with the fluorogenic substrate Z-YVAD-AFC (caspase-1 substrate VI, Calbiochem) as previously described (24). Briefly, cells were lysed in hypotonic cell lysis buffer (25 mM HEPES, 5 mM EGTA, 5 mM dithiothreitol (DTT), pH 7.5) on ice for 5–10 min and centrifuged to remove the insoluble fraction (12,500 × *g*, 10 min). THP-1 lysates (50 μl) or recombinant caspase-1 (10 units/ml) was incubated with 50 μM YVAD-AFC and 50 μl of reaction buffer (0.2% CHAPS, 0.2 M HEPES, 20% sucrose, 29 mM DTT, pH 7.5) for 2 h. After incubation, the fluorescence of the AFC released from the Z-YVAD-AFC substrate was measured by an increase in fluorescence (excitation 335 nm, emission 460 nm).

Statistical Analysis—Statistical analyses were performed using GraphPad Prism Version 5.00 for Windows (GraphPad Software). Differences between three or more groups were identified using one-way analysis of variance with post-hoc Bonferroni multiple comparison tests. Data are expressed as the mean ± S.D. from the number of assays indicated. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

RESULTS

IL-1β Release Is Inhibited by Eeyarestatin I—ESI (20) is a small molecule inhibitor of endoplasmic reticulum-associated protein degradation that works by inhibiting deubiquitination processes (25). ESI also induces an ER stress response by inhibiting protein translocation (20, 26). ESI contains a nitrofuran-containing group that is the functional domain responsible for its inhibitory action and an aromatic domain that has no inhibitory activity but targets the molecule to intracellular membranes (27). Incubation of LPS-primed murine peritoneal macrophages with ESI at both 10 and 50 μM, but not ESR35 composed of only the inactive aromatic domain (26), blocked ATP-induced release of IL-1β (Fig. 1, *A* and *B*) and IL-1α (supplemental Fig. S1*A*). ATP activates the cell surface P2X7 receptor and induces release of IL-1 via activation of the NLRP3 inflammasome (28–30). To test whether ESI blocks P2X7-dependent signaling rather than a general inhibition of the NLRP3

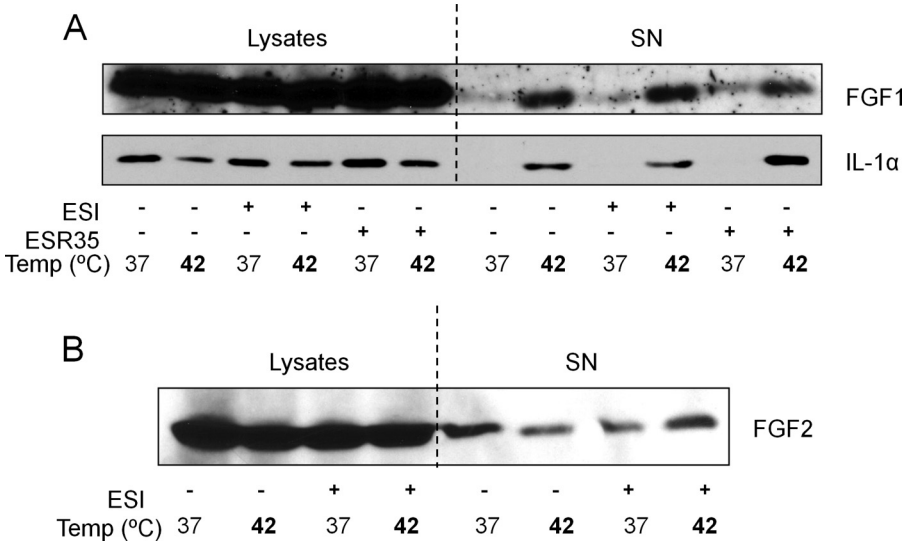

FIGURE 2. **Eeyarestatin I is not a general inhibitor of non-conventional protein secretion.** NIH3T3 cells were adenovirally transduced to express FGF1:HA, FGF2:HA, or mature IL-1α:V5. 48 h later the cells were subjected to a heat shock by incubation of the cells at 37 or 42 °C for 110 min in the presence or absence of 25 μM ESI or ESR35 with the levels of FGF1 (*A*), IL-1α (*A*), or FGF2 (*B*) in the lysates (*left side blots*) and the supernatants (*SN*, *right hand blots*) measured as described under "Experimental Procedures."

inflammasome, we tested the effects of the inhibitor against the potassium ionophore nigericin that induces activation of the NLRP3 inflammasome independently of the P2X7 receptor (29, 30). As with ATP, preincubation of LPS-primed macrophages with ESI inhibited nigericin-induced processing and release of IL-1β (Fig. 1, *C* and *D*) and IL-1α (supplemental Fig. S1*B*). These effects on IL-1 release were not due to decreased levels of cytokine expression as indicated by Western blots of the cell lysates (supplemental Fig. S1*C*). ESI also inhibited nigericin-induced IL-1β release from human monocytic THP-1 cells (Fig. 1, *E* and *F*), indicating that this is a mechanism common to both murine and human macrophages. No increase in cell death was detected by incubation with these compounds alone, indicating that they are not cytotoxic under these experimental conditions (supplemental Fig. S2, *A–C*). However, ESI but not ESR35, decreased the levels of cell death induced by nigericin in both peritoneal macrophages and THP-1 cells (supplemental Fig. S2, *A–C*).

IL-1α, like IL-1β, lacks a signal peptide for direction to the classical secretory pathway through the ER and the Golgi, and thus both cytokines harness non-conventional protein secretion pathways (7). Pro-IL-1α is not a substrate for caspase-1, and yet IL-1α release is inhibited in macrophages isolated from caspase-1 and NLRP3 KO mice in response to damage-associated molecular patterns (31), suggesting that IL-1α and IL-1β share a common secretory route in these systems. However, non-conventional secretion is not unique to IL-1 family members, and there are other secreted proteins such as FGF-1 and FGF-2 that harness such pathways. FGF-1 and mature IL-1α overexpressed in cell lines are secreted through a membrane translocation process in response to cellular stress such as heat shock, whereas FGF-2 is constitutively secreted (32). To study whether ESI has a direct effect on these translocation processes, we tested whether it blocked heat shock-induced export of FGF-1 and IL-1α (Fig. 2*A*) and/or constitutive export of FGF-2 (Fig. 2*B*) in NIH3T3 cells overexpressing FGF1, FGF2, or mature IL-1α. ESI had no effect on the secretion of these proteins (Fig. 2). These data suggest that ESI does not block non-conventional secretion *per se* but inhibits a caspase-1-dependent process.

DUBs Regulate Caspase-1 Activation and IL-1β Release—To further investigate whether the ESIs previously reported effect on protein translocation contributed to its inhibitory effect on IL-1 release or whether it was due to an inhibition of DUBs (25, 26), we investigated the effects of a selective DUB inhibitor b-AP15 and of the protein translocation inhibitor cpd A. b-AP15 is a small molecule DUB inhibitor of the proteasome-associated DUBs UCH37 and USP14 (33, 34), whereas cpd A is a selective inhibitor of the ER translocon with no effect on DUB activity (35). Pretreatment with b-AP15, but not with cpd A, inhibited ATP-induced IL-1β release from LPS-primed peritoneal macrophages (Fig. 3, *A* and *B*) and nigericin-induced release from LPS-primed THP-1 cells (Fig. 3, *C* and *D*). Neither b-AP15 nor cpd A was cytotoxic at the concentrations used in the assay (supplemental Fig. S2, *D* and *E*). As seen with ESI, b-AP15 reduced the levels of cell death induced by nigericin treatment in THP-1 cells, whereas cpd A had no effect (supplemental Fig. S2*E*). The potential involvement of DUBs in IL-1β release was further examined with a third DUB inhibitor, WP1130, that directly blocks the DUB activity of USP14, UCH37, USP5, and USP9x (36). WP1130 was also very recently reported to inhibit the activation of caspase-1 in response to NLRP3 inflammasome-activating stimuli (19). Treatment of murine peritoneal macrophages with WP1130 before ATP (Fig. 4, *A* and *B*) or of human THP1 cells before nigericin (Fig. 4, *C* and *D*) blocked release of IL-1β, in agreement with its reported effects on caspase-1 (19).

Because b-AP15 is known to inhibit two of the three DUBs associated with the proteasome, UCH37 and USP14 (34), and WP1130 also inhibits these enzymes, we decided to investigate whether they were involved in caspase-1-dependent release of IL-1β. We tested IU1, a specific inhibitor of USP14 (37). How-

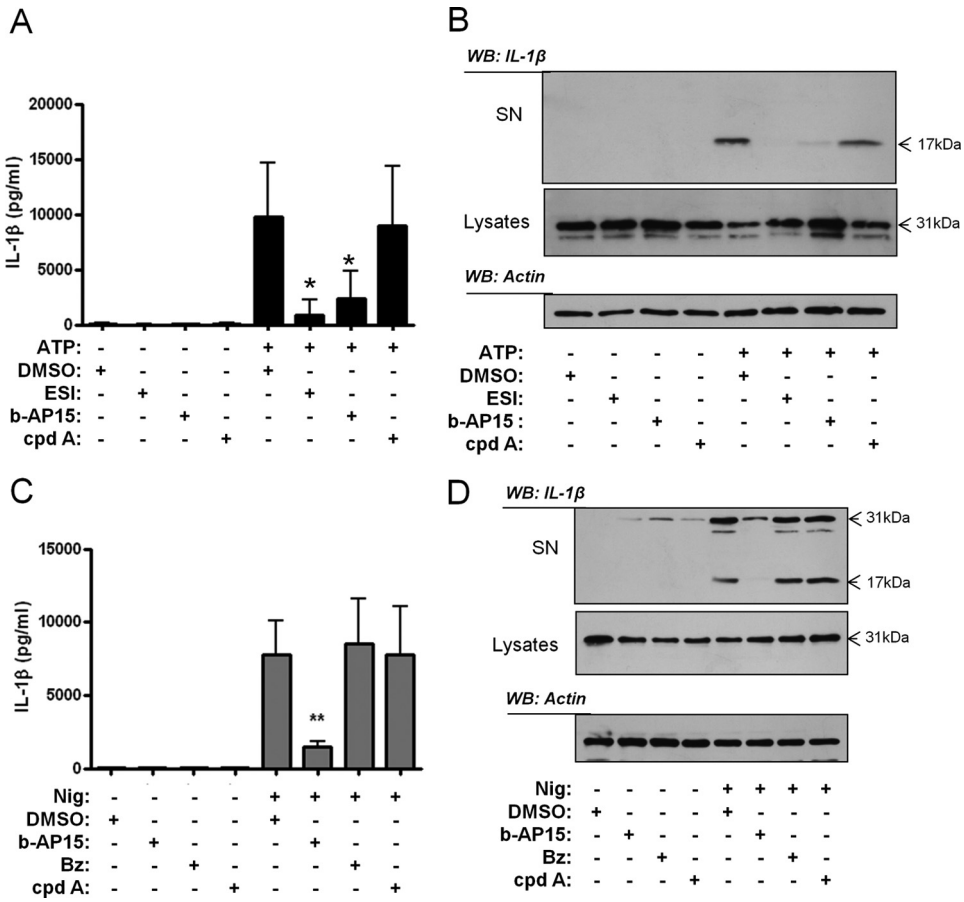

FIGURE 3. **Effects of ESI can be attributed to an inhibition of DUBs.** LPS-primed (1 μg/ml, 2 h) peritoneal macrophages (*black columns*) were incubated with 0.5% DMSO, ESI (50 μM), b-AP15 (1 μM), or cpd A (10 μM) 15 min before a 20-min incubation with 5 mM ATP (*A* and *B*). LPS-primed (1 μg/ml, 4 h) THP-1 cells (*gray columns*) were incubated with ESI (50 μM), b-AP15 (1 μM), bortezomib (*Bz*, 50 nM), or cpd A (10 μM) for 15 min before a 1-h incubation with nigericin (*Nig*, 10 μM) (*C* and *D*). Supernatants (*SN*) and cell lysates were collected and analyzed for IL-1β by ELISA (*A* and *C*) and Western blot (*B* and *D*). In the blots (*B* and *D*) the band at 31 kDa represents pro-IL-1β, and the band at 17 kDa represents mature IL-1β. Also shown is the β-actin. Data are presented as the mean of three separate experiments ± S.D. **, $p < 0.01$; *, $p < 0.05$. All inhibitor groups were compared with ATP or nigericin alone.

ever, IU1 had no effect on ATP (Fig. 4, *A* and *B*) or nigericin-induced (Fig. 4, *C* and *D*) IL-1β release. The lack of effect of IU1 suggested that the enzyme responsible for the observed effect could be UCH37. However, silencing of UCH37 (60% reduction compared with untreated control) (Fig. 4, *E* and *F*) had no effect on nigericin-induced IL-1β release from THP-1 cells (Fig. 4*G*). These data and the fact that UCH37 or USP14 may have a redundant DUB function (34, 38) and also that b-AP15 and WP1130 inhibit both UCH37 and USP14 suggests that a contribution from both enzymes may be needed. However, USP14 inhibition with IU1 after UCH37 silencing in THP-1 again had no effect on the levels of IL-1β released in response to nigericin (Fig. 4*H*). These data suggest that multiple or as yet uncharacterized DUBs are required for IL-1β processing and release.

DUB Inhibitors Block IL-1β Release Independently of the Proteasome—Because ESI, WP1130, and b-AP15 cause an increase in polyubiquitinated proteasomal substrates (33, 36, 39) similar to that obtained after proteasome inhibition, we next investigated whether the inhibitory effects of these molecules on IL-1β release could be explained by an effect on proteasomal function. LPS-primed mouse peritoneal macrophages were treated with the proteasome inhibitors bortezomib or MG262, and the effects on ATP-induced IL-1β release observed. Neither compound affected the release of IL-1β (Fig. 5, *A* and *B*) or cell death (supplemental Fig. S2*F*) at doses known to inhibit the proteasome (40). Similarly, treatment of THP-1 cells with bortezomib had no effect on IL-1β release induced by nigericin (Fig. 3, *C* and *D*) or cell death (supplemental Fig. S2*E*). These data suggest that the effects of the DUB inhibitors on IL-1β release were not due to an inhibition of proteasome function.

DUBs Are Required for ASC Oligomerization—We next investigated whether the effect of DUB inhibitors on IL-1β release was due to a direct inhibition of caspase-1. However, ESI or b-AP15 had no direct effect on caspase-1 activity as measured by the cleavage of a fluorogenic caspase-1 substrate (Z-YVAD-AFC) by either recombinant caspase-1 (Fig. 6, *A* and *B*) or a hypotonic THP-1 cell lysate (Fig. 6*C*). We used the caspase-1 inhibitor YVAD as a positive control to directly inhibit caspase-1 (Fig. 6, *A*, *B*, and *C*).

ASC is an adaptor protein required for the activation of the NLRP3 and AIM2 inflammasomes (4, 6), and upon inflammasome assembly its presence within the complex is readily visualized inside cells by its oligomerization and the appearance of large aggregates called specks (8, 33, 41). Pretreatment of LPS-primed THP-1 cells with b-AP15 significantly reduced the

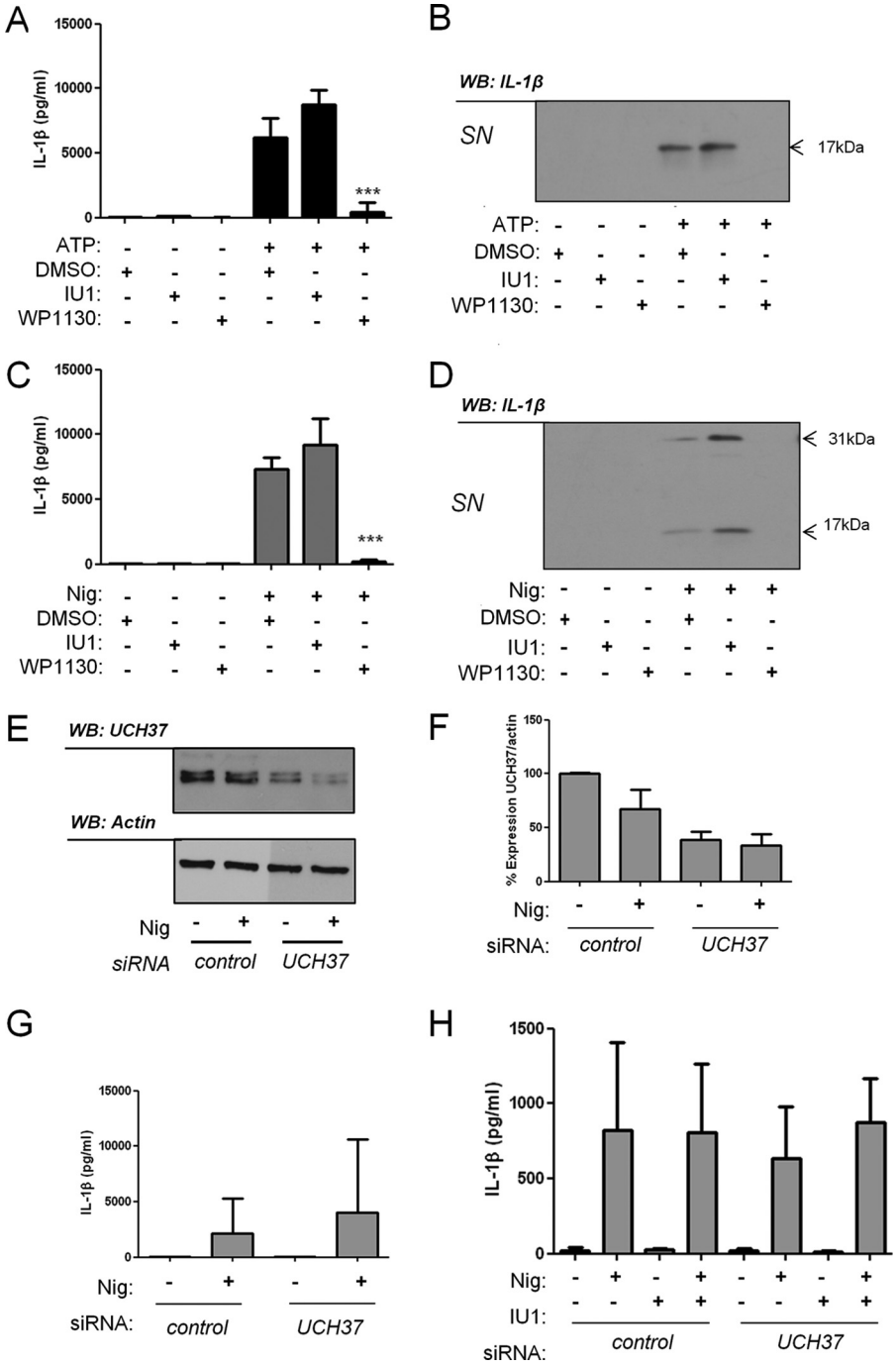

FIGURE 4. **Identification of the DUB regulating IL-1β release.** LPS-primed (1 μg/ml, 2 h) peritoneal macrophages (*black bars*) were incubated with 0.5% DMSO, WP1130 (1 μM), or IU1 (50 μM) 15 min before a 20-min incubation with 5 mM ATP (*A* and *B*). *WB*, Western blot. LPS-primed (1 μg/ml, 4 h) THP-1 cells (*gray bars*) were incubated with 0.5% DMSO, WP1130 (1 μM), or IU1 (50 μM) 15 min before a 1-h incubation with nigericin (10 μM) (*C* and *D*). Supernatants (*SN*) were collected and analyzed for IL-1β by ELISA (*A* and *C*) and by Western blot (*B* and *D*); the band at 31 kDa represents pro-IL-1β, and the band at 17 kDa represents mature IL-1β. THP-1 cells were treated with control or UCH37 siRNA (1 μM) for 72 h (*E–H*). These THP-1 cells were then primed with LPS (1 μg/ml, 4 h) followed by treatment with nigericin (*Nig*, 10 μM, 1 h) (*E–H*). In *H*, half of the LPS-treated THP-1 cells received an incubation with IU1 (50 μM, 15 min) before nigericin treatment. Levels of UCH37 in the cell lysates were analyzed by Western blot (*E*) and compared relative to actin by densitometry (*F*). IL-1β release was measured by ELISA (*G* and *H*). Blots are representative of three experiments. Pooled ELISA data are presented as the mean ± S.D. from three (*A*, *C*, and *G*) and two (*H*) experiments. *, $p < 0.05$; ***, $p < 0.001$ *versus* ATP or nigericin alone (*A* and *C*) or *versus* untreated control (*F*).

numbers of ASC specks formed after nigericin treatment (Fig. 7, *A* and *C*). Similarly, ATP-induced speck formation in murine peritoneal macrophages was also inhibited by b-AP15 (Fig. 7 *B* and *D*), suggesting that DUB inhibitors act upstream of caspase-1 for the secretion of IL-1β. To further demonstrate this we analyzed the presence of ASC oligomers by Western blot as previously described (22). We found that ASC oligomers were only present in THP-1 cells that had not been incubated with ESI, b-AP15, or WP1130 before nigericin treatment (Fig. 7*E*). These DUB inhibitors also inhibited the release of the processed caspase-1 p10 subunit, correlating with reduced IL-1β secretion (Fig. 7*F*). Altogether these data demonstrate that the

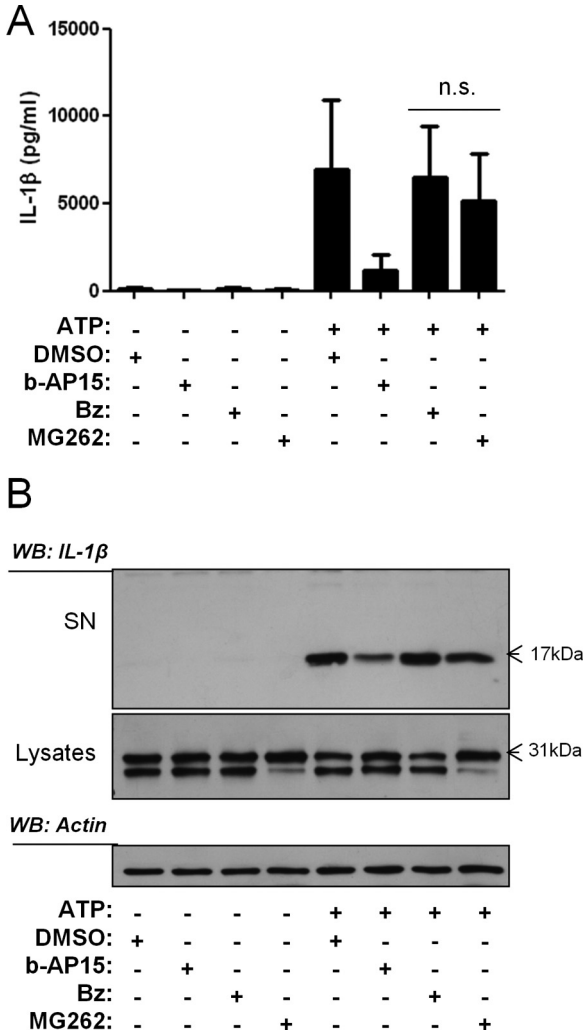

FIGURE 5. **DUB inhibitors block IL-1β release independently of the proteasome.** LPS-primed (1 μg/ml, 2 h) peritoneal macrophages were incubated with 0.5% DMSO, b-AP15 (1 μM), and the proteasome inhibitors bortezomib (*Bz*, 50 nM) or MG262 (30 μM) for 15 min before a 20-min incubation with 5 mM ATP. Levels of IL-1β released into the supernatants (*SN*) were measured by ELISA (*A*) and by Western blot (*WB*, *B*). Also shown in *B*, are blots for IL-1β and actin in the cell lysates. Blots are representative of three separate experiments. ELISA data are the mean ± S.D. of three separate experiments. *n.s.*, no significant when compare with ATP alone.

effects of these DUB inhibitors on IL-1β release occur upstream of caspase-1 by blocking the oligomerization of ASC.

NLRP3 (and Partially AIM2) Inflammasome-dependent IL-1β Release Require DUBs—Given the inhibitory effect of b-AP15 on ASC oligomerization and the requirement of this adaptor protein to activate the AIM-2 and NLRP3 inflammasomes (4, 6), we decided to investigate whether DUB inhibition targeted one or both of these inflammasomes. ATP and nigericin are well established activators of the NLRP3 inflammasome (29). To further confirm the requirement of DUBs for NLRP3 inflammasome activation, LPS-primed macrophages were treated with another well characterized NLRP3 activator, crystals of MSU (42). b-AP15 and WP1130 both inhibited MSU-induced IL-1β release, further confirming that DUBs are involved in the activation of the NLRP3 inflammasome (Fig. 8, *A* and *B*). The AIM2 inflammasome is activated by cytosolic double-stranded DNA (4, 6), and so to determine whether AIM2 inflammasome formation was also DUB dependent we transfected LPS-primed macrophages from NLRP3 KO mice with plasmid DNA in the presence or absence of b-AP15. We observed a partial inhibition of IL-1β release induced by DNA transfection by b-AP15 (Fig. 8, *C* and *D*), indicating that DUBs are also involved in AIM-2 inflammasome activation, although to a lesser extent than for NLRP3.

Autophagy is a catabolic process in which ubiquitination plays an important role (43) and is also reported to contribute to the regulation of the inflammasome under certain conditions (44, 45). Microtubule-associated protein 1 light chain 3 (LC3) participates in autophagosome formation and maturation (46). LC3 KO animals have decreased levels of autophagy that result in increased caspase-1 activation and IL-1β release (44). In addition, it was recently reported that ASC can be ubiquitinated and targeted for autophagy as a means of regulating inflammasome activity (45). LC3 is present in two different forms: LC3-I (18 kDa), that is cytosolic, and LC3-II (16 kDa), which associates with the autophagosomal membrane and whose levels correlate with the autophagosome numbers within the cell (47). In LPS-primed THP-1 cells nigericin treatment induced an increase of the levels of LC3-II, indicating an increase in autophagy, which was not affected by treatment with b-AP15 (Fig. 8*E*). Furthermore, b-AP15 in the absence of nigericin treatment did not affect levels of LC3-II compared with untreated cells (Fig. 8*E*) despite an overall increase in poly ubiquitinated proteins (Fig. 8*F*).

DISCUSSION

In this study we have shown that inhibition of DUBs with the inhibitors ESI, b-AP15, or WP1130 blocked IL-1β processing and release induced by NLRP3-inflammasome-dependent responses with a partial effect observed on an AIM2-inflammasome-dependent response. The effects we observe with the DUB inhibitors are in complete agreement with a recent study that reported WP1130 to effectively inhibit NLRP3-dependent activation of caspase-1 (19). Our data suggest that these effects were upstream of inflammasome formation as DUB inhibitors blocked ASC speck formation but had no direct effect on caspase-1 activity. Together with recently published data (19), our data provide new insights into the signaling mechanisms regulating inflammasome formation.

Ubiquitination, a post-translational modification of proteins that regulates many biological processes, has a well established role in regulating immune responses. Activation of pattern recognition receptors such as Toll-like receptors, Rig-1-like receptors, and NLR triggers different signaling cascades that culminate with the synthesis of pro-inflammatory cytokines and/or interferons (48). The ubiquitin ligase TRAF6 (TNF receptor-associated factor) plays an essential role in Toll-like receptor-dependent activation of NFκB and mitogen-activated protein kinase (48). In the case of Rig-1-like receptors, RIG-1 requires ubiquitination of one of its CARD domains (caspase recruitment domain) to signal effectively (49), and NLR family member NOD2-mediated activation of NFκB and mitogen-activated protein kinases also requires ubiquitination of the protein kinase RIP2 by cIAP or TNF receptor-associated factor proteins (50, 51). Together these studies show that ubiquitination is

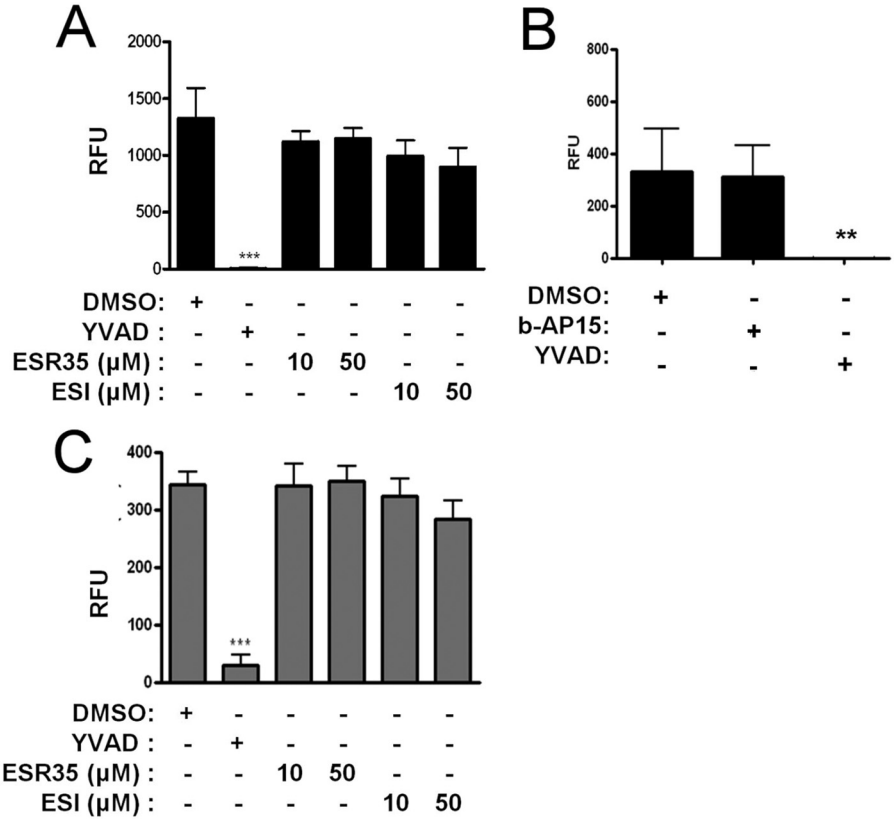

FIGURE 6. **Caspase-1 activity is not blocked by DUB inhibitors.** Recombinant caspase-1 (10 units/ml) was incubated with 0.5% DMSO, YVAD (100 μM), ESI, or ESR35 (10 or 50 μM) (*A*) or 0.5% DMSO, YVAD (100 μM) or b-AP15 (1 μM) (*B*) for 10 min before the addition of the fluorogenic substrate (Z-YVAD-AFC). Caspase-1 activity was measured after 2 h of incubation at 37 °C. Data are expressed as relative fluorescent units (*RFU*). *C*, THP-1 cells lysates were incubated with 0.5% DMSO, YVAD (100 μM), ESI, or ESR35 (10 or 50 μM) for 10 min before the addition of the fluorogenic substrate (Z-YVAD-AFC). Caspase-1 activity was measured after 2 h of incubation at 37 °C. The data are representative of three experiments and are presented as the mean ± S.D. **, $p < 0.01$; ***, $p < 0.001$ *versus* DMSO-treated.

commonly present in immune signaling pathways where it acts as a signal to recruit ubiquitin-binding proteins regulating the assembly of signaling complexes. As with any dynamic process, assembly of ubiquitin chains has to occur in a regulated manner involving their disassembly; hence, the importance of DUBs in these systems. The best studied DUBs that regulate immune responses are A20 (also known as tumor necrosis factor α-induced protein 3, TNFAIP3) and CYLD (cylindromatosis deubiquitinase) (52). These DUBs specifically cleave Lys-63-linked polyubiquitin chains and negatively regulate NFκB signaling (53).

There is evidence that caspase-1 activation within the inflammasome is regulated by ubiquitin, and recently ubiquitin ligases of the IAP family were shown to regulate the processing and release of IL-1β (16, 17). Sug1, a regulatory protein of the 26 S proteasome with ubiquitin ligase activity mediates ubiquitination of NLRC4 (54), and the ubiquitin ligase-associated protein SGT1 interacts with the NLRP3 inflammasome regulating its activity (18). Recent findings have reported that deubiquitination of NLRP3 is important for its activation (19). This is in agreement with the data reported in this manuscript. We provide additional insights, however, that there is also a partial DUB dependence of AIM2 inflammasome formation, that the effects of DUBs occur upstream of ASC oligomerization, and that the effects on IL-1 secretion are specific to caspase-1-dependent processes rather than an effect on non-classical protein secretion.

Although different release mechanisms for IL-1β have been proposed, the exact pathways through which it exits the cell remain unknown (7). Like IL-1β and IL-1α, there are multiple other proteins that lack a signal peptide and that harness nonconventional routes of secretion, such as FGF-1, FGF-2, or galectin-1 (55). Cellular stress such as heat shock induces the release of FGF-1 and IL-1α via translocation across the plasma membrane as part of a complex containing S10013 (55, 56). FGF-2 secretion, however, occurs constitutively and is mediated by phosphatidylinositol 4,5-bisphosphate at the inner leaflet and by heparan sulfate proteoglycans at the extracellular side of the plasma membrane (57). The direct membrane translocation of FGF-1 and -2 or mature IL-1α, when overexpressed in NIH3T3 cells, was not inhibited by ESI (Fig. 2). This suggested that the effect of ESI on IL-1β secretion from macrophages was selective for the pathway regulating caspase-1 activity. Known ESI targets include Sec61-mediated translocation of the ER and DUB activity associated with the endoplasmic reticulum-associated protein degradation process (26, 39, 58). IL-1β lacks a signal peptide, and its secretion is not blocked by brefeldin A (59), suggesting that it is highly unlikely that the effects of ESI reported here reflected an effect on ER translocation. We provided additional evidence for this by showing that the selec-

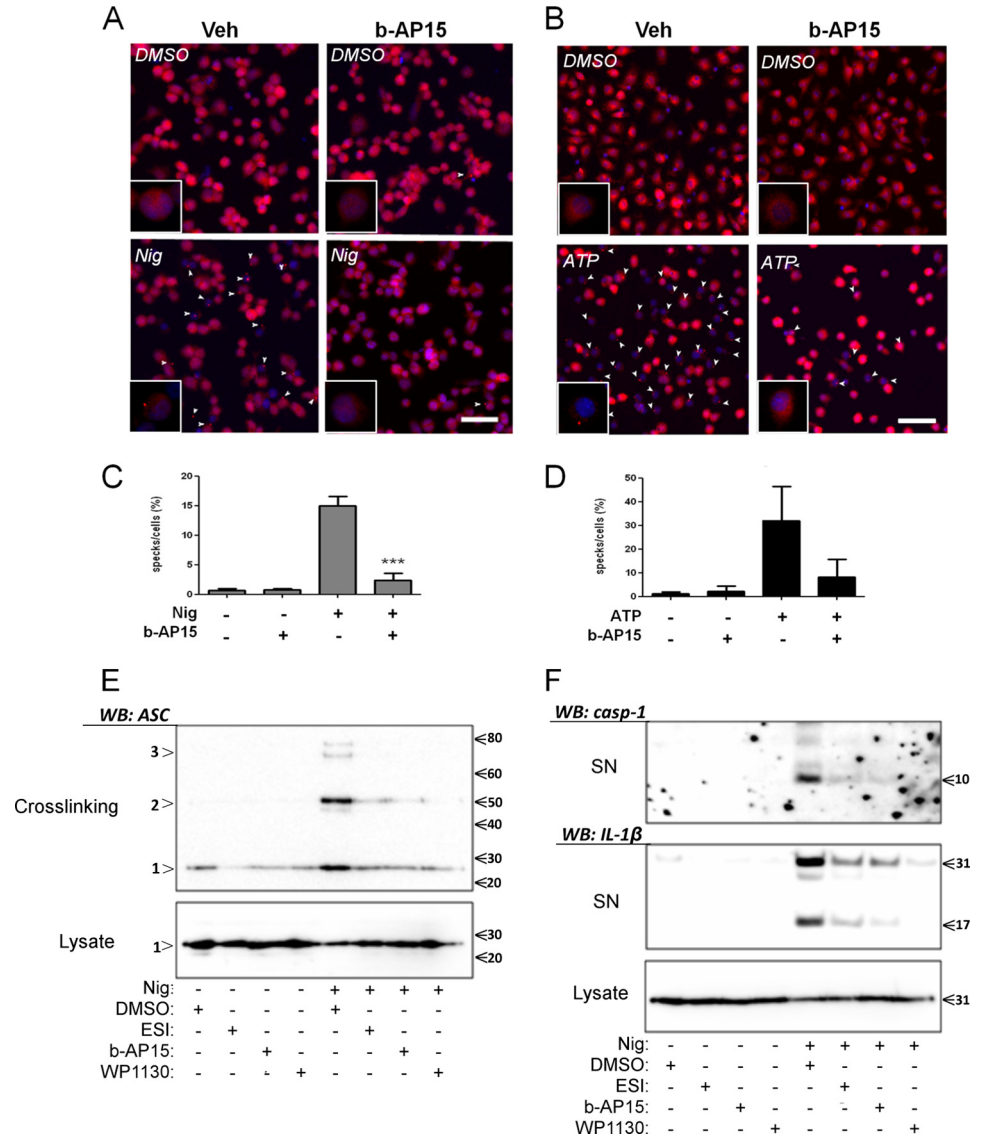

FIGURE 7. **ASC oligomerization is blocked by DUB inhibitors.** LPS-primed (1 μg/ml, 4 h) THP-1 cells were incubated with 0.5% DMSO or b-AP15 (1 μM) 15 min before a 1-h incubation with nigericin (*Nig*, 10 μM). Cells were fixed and analyzed for ASC expression (*A*), and the number of ASC specks was quantified and expressed as the percentage of specks per cell number (*C*, *gray columns*), $n = 3$. LPS-primed (1 μg/ml, 2 h) peritoneal macrophages were incubated with 0.5% DMSO or b-AP15 (1 μM) 15 min before a 20-min incubation with ATP (5 mM). Cells were fixed and analyzed for ASC expression (*B*), and the number of ASC specks was quantified and expressed as the percentage of specks per cell number (*D*, *black columns*). LPS-primed (1 μg/ml, 4 h) THP-1 cells were incubated with 0.5% DMSO, ESI (50 μM), b-AP15 (1 μM), or WP1330 (1 μM) for 15 min before a 1-h incubation with nigericin (*Nig*, 10 μM). Cells were lysed and analyzed for the presence of ASC oligomers (*Cross-linking*) (*E*). *1>*, *2>*, and *3>* indicate the ASC monomer, dimer, and trimer, respectively. Release of active processed caspase-1 p10 subunit was detected in concentrated supernatant (*SN*) by Western blot (*WB*), as was IL-1β (*F*). Levels of ASC (*E*) and pro-IL-1β (*F*) in the cell lysate are also shown. The data are representative of three (*A* and *C*) and two (*B*, *D*, *E*, and *F*) experiments. The data are presented as the mean ± S.D. ***, $p < 0.001$ *versus* nigericin treatment (*C*). *Scale bar* = 50 μm.

tive ER translocation inhibitor cpd A (35) had no effect on IL-1β processing and release (Fig. 3), suggesting the effect of ESI on IL-1 release was DUB-mediated. The DUB dependence of caspase-1-dependent IL-1 secretion was further confirmed by use of two more selective DUB inhibitors, b-AP15 and WP1130.

The inhibitor activity of b-AP15, tested on a panel of different DUBs, is selective for UCH37 and USP14 (34). DUBs not inhibited by b-AP15 include BAP1, a deubiquitinase from the same family as UCH37 and that shares a high degree of similarity (34, 60). b-AP15 also failed to block total DUB activity from cell lysates measured by cleavage of the substrate ubiquitin-amidomethylcoumarin, whereas *N*-ethylmaleimide did (data not shown) (34), confirming that b-AP15 is not a pan-DUB inhibitor. However, inhibition of USP14 activity with IU1, silencing of UCH37 with siRNA, or a combination of both approaches to inhibit both USP14 and UCH37 simultaneously did not inhibit the release of IL-1β (Fig. 4). It is possible that the level of UCH37 silencing we achieved with siRNA may not have been sufficient to observe a measurable effect on nigericin-induced IL-1β release. However, we cannot rule out the possibility that there is further redundancy with other DUBs throughout this system and/or that these inhibitors act on more than one target.

Although AIM2 inflammasome activation is restricted to DNA sensing, NLRP3-dependent caspase-1 activation can be induced by structurally diverse stimuli such as ATP, nigericin,

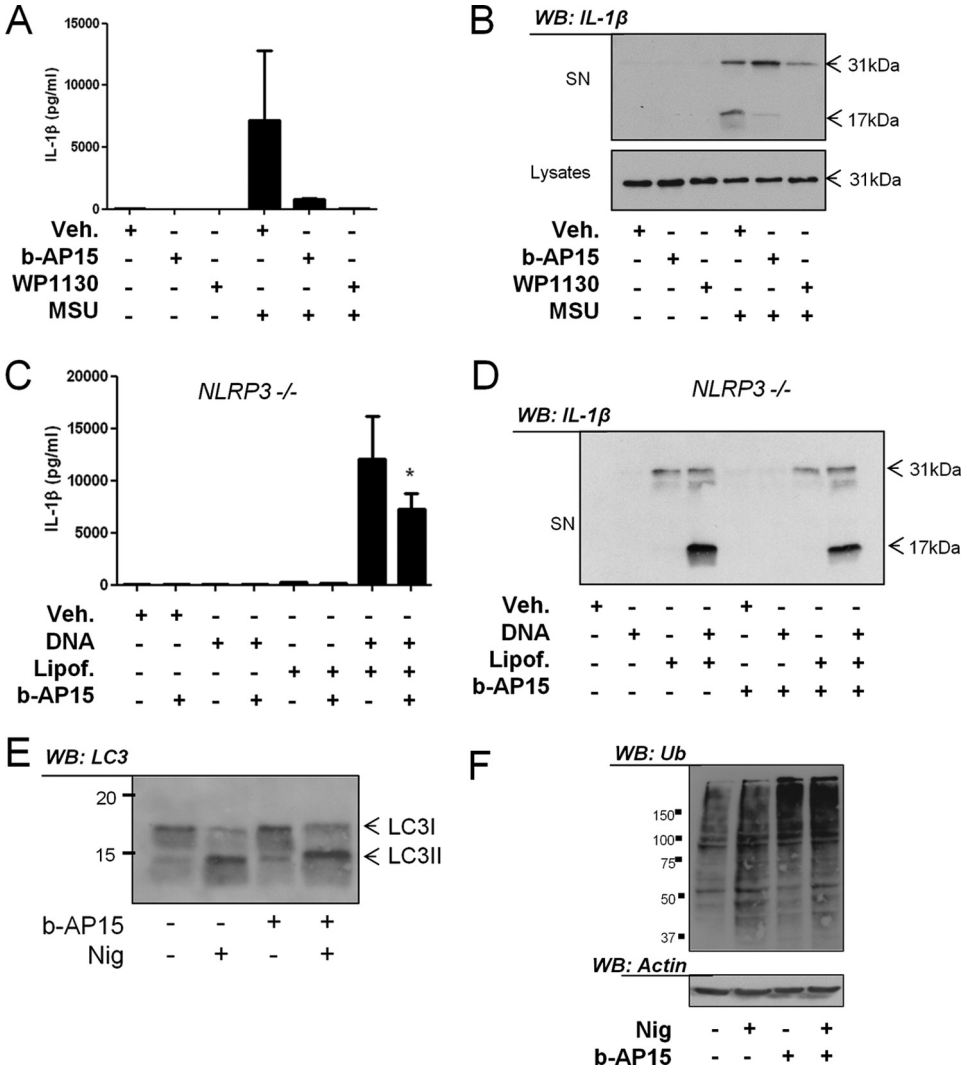

FIGURE 8. **Inflammasome dependence of DUB activity.** LPS-primed (1 μg/ml, 4 h) THP-1 cells were incubated with 0.5% DMSO, b-AP15 (1 μM), or WP1130 (1 μM) 15 min before a 1-h incubation with MSU (250 μg/ml). IL-1β release in the supernatants (*SN*) was measured by ELISA (*A*) and Western blot (*WB*, *B*). Data are presented as the mean ± S.D. of three separate experiments (*A*) or are representative (*B*). LPS-primed peritoneal macrophages from NLRP3 KO mice (*C* and *D*) were pretreated with b-AP15 (1 μM, 15 min) before activation with DNA (1.6 μg/ml), Lipofectamine (*Lipof.*), or both together. IL-1β released into the supernatants (*SN*) was measured by ELISA (*C*) and Western blot (*D*). Data are presented as the mean ± S.D. of three separate experiments (*C*) or are representative (*D*). *, $p < 0.05$ *versus* DNA/lipofectamine treatment. LPS-primed (1 μg/ml, 4 h) THP-1 cells were incubated with b-AP15 (1 μM) 15 min before a 1-h incubation with nigericin (10 μM). The cell lysates were recovered and blotted for the autophagy marker LC3 (*E*) and ubiquitin (*F*). In *E* both LC3I and LC3II are indicated. The blots in *E* and *F* are representative of three separate experiments.

or MSU (4, 6). Inhibition of protein synthesis with ricin or other ribosomal inhibitors stimulates NLPR3-dependent IL-1β release in cells that have been previously treated with LPS, possibly due to the disappearance of a protein/s required for inflammasome regulation, and this can be blocked by the proteasomal inhibitors bortezomib and MG-132 (61, 62). Furthermore, nigericin, ATP, and MSU all reduce protein translation, suggesting that inhibition of protein synthesis may be the mechanism regulating activation of the NLRP3 inflammasome (62). However, ATP- and nigericin-induced IL-1β release has also been shown to occur independently of proteasome inhibition with MG-132 (63, 64). Consistent with these latter findings, we report here that both ATP- and nigericin-induced IL-1β release was not affected by proteasome inhibitors, indicating that the effects of DUB inhibition on IL-1β release do not involve proteasomal activity. Thus the data presented here suggest DUBs regulate signaling processes required for the assembly of the inflammasome and caspase-1 activation.

Another mechanism through which inflammasome activity can be regulated is autophagy (65). Ubiquitinated proteins can be recruited into the autophagic route by the adaptor proteins p62 (also called SQSTM1/A170) (46) through its interaction with the autophagosomal protein LC3 that results in the engulfing and degradation of p62 and its ubiquitinated cargo (66). Activation of the inflammasome with nigericin or DNA is accompanied by an increase in autophagy within macrophages (45). This activation leads to the ubiquitination of ASC and possibly other inflammasome components that can then bind p62 entering the autophagy route of degradation (45). We found, however, no evidence linking the effects of the DUB inhibitors on IL-1β release to changes in levels of autophagy within the cells (Fig. 8). DUB inhibitors did, however, inhibit

ASC oligomerization and consequently caspase-1 activation but had no direct effect on the activity of recombinant caspase-1 (Figs. 6 and 7), suggesting that the process regulated by DUBs occurs upstream of inflammasome assembly. It could be possible that DUB inhibitors induce the accumulation of ubiquitinated inflammasome components and consequently increase their degradation by autophagy without altering the autophagy levels in the macrophage. Alternatively it is possible that DUB inhibitors block DUB-mediated alterations of the polyubiquitin chain of inflammasome components that are required for the assembly of the complex and consequently caspase-1 activation and IL-1β release as suggested recently (19). In conclusion, we contribute to the growing evidence that the ubiquitin system plays a crucial role in inflammasome activation. Furthermore, in addition to their effects on cancer cells, the DUB inhibitors used in this study could also represent a new class of anti-inflammatory molecule and may be effective in IL-1-dependent pathologies.

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