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Multiple Cathepsins Promote Pro–IL-1β Synthesis and NLRP3-Mediated IL-1β Activation

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Sterile particles induce robust inflammatory responses that underlie the pathogenesis of diseases like silicosis, gout, and atherosclerosis. A key cytokine mediating this response is IL-1β. The generation of bioactive IL-1β by sterile particles is mediated by the NOD-like receptor containing a pyrin domain 3 (NLRP3) inflammasome, although exactly how this occurs is incompletely resolved. Prior studies have found that the cathepsin B inhibitor, Ca074Me, suppresses this response, supporting a model whereby ingested particles disrupt lysosomes and release cathepsin B into the cytosol, somehow activating NLRP3. However, reports that cathepsin B-deficient macrophages have no defect in particle-induced IL-1β generation have questioned cathepsin B's involvement. In this study, we examine the hypothesis that multiple redundant cathepsins (not just cathepsin B) mediate this process by evaluating IL-1β generation in murine macrophages, singly or multiply deficient in cathepsins B, L, C, S and X. Using an activity-based probe, we measure specific cathepsin activity in living cells, documenting compensatory changes in cathepsin-deficient cells, and Ca074Me's dose-dependent cathepsin inhibition profile is analyzed in parallel with its suppression of particle-induced IL-1β secretion. Also, we evaluate endogenous cathepsin inhibitors cystatins C and B. Surprisingly, we find that multiple redundant cathepsins, inhibited by Ca074Me and cystatins, promote pro–IL-1β synthesis, and to our knowledge, we provide the first evidence that cathepsin X plays a nonredundant role in nonparticulate NLRP3 activation. Finally, we find cathepsin inhibitors selectively block particle-induced NLRP3 activation, independently of suppressing pro–IL-1β synthesis. Altogether, we demonstrate that both small molecule and endogenous cathepsin inhibitors suppress particle-induced IL-1β secretion, implicating roles for multiple cathepsins in both pro–IL-1β synthesis and NLRP3 activation. *The Journal of Immunology*, **2015, 195: 1685–1697.**

Sterile particles induce robust inflammatory responses that underlie the pathogenesis of many diseases. These pathogenic particles are diverse and include silica (1–4), which causes silicosis, monosodium urate (5), the etiologic agent in gout, and cholesterol crystals (CC) (6, 7), which are thought to contribute to the pathogenesis of atherosclerosis. Importantly, the sterile inflammatory response and resultant diseases caused by these particles all involve signaling through the IL-1R, IL-1R1 (8, 9). Although IL-1R1 can be stimulated by either of two cytokines, IL-1α or IL-1β, it has been shown that IL-1β plays a pivotal role in disease pathogenesis (10) because it not only directly stimulates IL-1R1–dependent inflammatory signaling but is also needed for the secretion of IL-1α from cells (11). Therefore, it is important to understand the exact mechanisms underlying the generation and secretion of active IL-1β. However, this process is still incompletely understood and the focus of the present report.

The generation of biologically active IL-1β is highly regulated and usually proceeds in two distinct steps (12, 13). The first step (Signal 1 or "priming") is initiated when cells such as macrophages are stimulated by certain cytokines, pathogen-associated molecular patterns, or danger-associated molecular patterns. Signal 1 leads to the nuclear translocation of NF-κB, which then stimulates the synthesis of biologically inactive pro–IL-1β and, among other things, NOD-like receptor containing a pyrin domain 3 (NLRP3), a protein important for IL-1β activation. The second step (Signal 2 or "activation") induces the formation of a multimolecular complex, known as the inflammasome. Inflammasomes are composed of a sensor protein, an adaptor protein, apoptosis-associated speck-like protein containing a CARD, and an executioner protease, caspase-1. Each inflammasome sensor detects distinct stimuli, thereby initiating multimerization and activating caspase-1, which then cleaves pro–IL-1β and facilitates the secretion of bioactive mature IL-1β. Among the known inflammasomes, the NLRP3 inflammasome is unique. Although all inflammasomes rely on the availability of a newly synthesized pool of pro–IL-1β, basal levels of NLRP3 itself are limiting, making priming especially critical for de novo NLRP3 transcription and subsequent activation (14, 15). Moreover, the NLRP3 inflammasome is the exclusive mediator of IL-1β activation in response to sterile particles (1–7).

Although the NLRP3 inflammasome is located in the cytosol, how this intracellular complex senses the presence of extracellular particles has been of considerable interest. It has been shown that

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Abbreviations used in this article: CC, cholesterol crystal; CHX, cycloheximide; dAdT, poly(deoxyadenylic-deoxythymidylic) acid; LDH, lactate dehydrogenase; LLOMe, Leu-Leu-OMe; LMD, lysosomal membrane disruption; LRR, leucine-rich repeat; NLRP3, NOD-like receptor containing a pyrin domain 3; PM, peritoneal macrophage; qPCR, quantitative PCR; ROS, reactive oxygen species; siRNA, small interfering RNA; WT, wild-type.

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internalization of particles by phagocytosis is a first essential step in activating the NLRP3 inflammasome (2). Multiple mechanisms have been proposed as to how particles in phagosomes then lead to NLRP3 inflammasome activation, including lysosomal membrane disruption (LMD) (2, 3, 6, 7, 13, 16–29), potassium efflux (1, 4, 7, 21, 29–37), and the generation of reactive oxygen species (ROS) (1, 27, 29, 30, 32, 36, 38–40), among various other mechanisms (reviewed Ref. 12). All of these pathways may contribute to this process. In support of the LMD model, it has been shown that particles like silica, CC, and the adjuvant alum can cause LMD (2, 6, 7), leading to the leakage of the lysosomal cysteine protease cathepsin B into the cytosol, where this protease is thought to activate NLRP3 through an as yet undescribed mechanism. Consistent with this model, particle-induced activation of the NLRP3 inflammasome is blocked by inhibitors of lysosomal acidification (cathepsins are optimally active in acidic conditions) and inhibitors of cathepsin B. However, the requirement for cathepsin B in this process is controversial.

A role for cathepsin B in NLRP3 activation is supported by a number of studies showing that Ca074Me, an inhibitor reported to be specific for cathepsin B, suppresses IL-1β activation induced by particulate and nonparticulate stimuli (2, 7, 17, 20, 21, 25–29, 41–46). However, despite a few subsequent studies showing that cathepsin B– or L–deficient macrophages show partial impairment of this response (6, 25, 41), several follow-up studies have found that responses are intact in these same mutant cells (31, 42, 47). Thus, it has become unclear whether the efficacy of Ca074Me is really a result of cathepsin B inhibition or whether this is an off-target effect. Indeed, there are there are several reports demonstrating that Ca074Me inhibits other cathepsins as well (48–52). Therefore, one hypothesis proposed to explain the discrepancy between Ca074Me and genetic models is that multiple cathepsins, which are a highly conserved family of proteases, play redundant roles in NLRP3 activation (53). Redundancy of cathepsins B and L has been demonstrated in a mouse model, where deficiency of both results in neonatal mortality whereas deficiency of either alone does not (54). Similar redundancy has also been observed in mouse cancer models showing upregulation of cathepsin X when cathepsin B is knocked out (55). However, the role of redundant cathepsins has not been examined in the context of NLRP3 activation and remains an open question.

In this study, we use genetic inactivation of multiple cathepsins, together with exogenous and endogenous inhibitors of these proteases, and an activity-based probe to investigate the role of cathepsins in NLRP3-dependent particle-induced IL-1β secretion. This analysis reveals that multiple cathepsins indeed contribute to IL-1β secretion. Surprisingly, our data also demonstrate that cathepsins contribute not only to the inflammasome-mediated cleavage of pro–IL-1β into mature IL-1β (Signal 2) but also to the priming step of pro–IL-1β synthesis (Signal 1). In addition, we found a unique role for cathepsin X in nigericin-induced NLRP3 activation, a protease not previously implicated in the IL-1 response. Taken together, these data clarify the contribution of cathepsins to particle-induced IL-1β responses and define a previously unappreciated role for cathepsins and their inhibitors in regulating pro–IL-1β synthesis. In doing so, this study provides insight into the mechanistic regulation of IL-1β production and points to cathepsins as unique therapeutic targets for controlling particle-induced sterile inflammatory responses.

Materials and Methods

Reagents and Abs

Abs for Western blots were against mouse IL-1β (R&D Systems), caspase-1 p10 (sc-514; Santa Cruz Biotechnology), NLRP3 (Cryo2; Enzo Life Sciences), β-actin (C4; Santa Cruz Biotechnology), and GAPDH (6C5; EMD Millopore). ELISA kits were purchased for mouse IL-1β (BD Biosciences), pro–IL-1β, and TNF-α (eBioscience). Ultrapure LPS was from *Salmonella minnesota* (InvivoGen). Poly(deoxyadenylic-deoxythymidylic) acid and nigericin were purchased from Sigma-Aldrich (St. Louis, MO). Silica crystals (MIN- U-SIL 15) were obtained from U.S. Silica (Frederick, MD). Cholesterol crystals were synthesized by acetone supersaturation and cooling (6), Alum (Imject alum adjuvant; a mixture of aluminum hydroxide and magnesium hydroxide) was from Pierce Biotechnology, and Leu-Leu-OMe-HCl was from Chem-Impex International. ZVAD-FMK and Ca-074-Me were from Enzo Life Sciences, and K777 was initially gifted to us by S. A. Robertson and J. H. McKerrow at University of California (San Francisco, CA), and further stocks obtained through services from the National Heart, Lung, and Blood Institute's SMARTT Program. Lipofectamine 2000, RNAiMax, and all small interfering RNA (siRNA) smart pools were from Life Technologies and Endoporter was from Gene Tools.

Animal and cell lines

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. NLRP3-deficient mice (56) were provided by Millennium Pharmaceuticals. Cathepsin S–deficient mice (57) were provided by Dr. H. Chapman (University of California), cathepsin L–deficient (58), and cathepsin B–deficient (53) mice were provided by Dr. H. Ploegh (Harvard Medical School, Boston, MA), cathepsin C–deficient mice (59) were provided by Dr. Christine Pham (Washington University School of Medicine, St. Louis), and all mice have been backcrossed to C57BL/6 background. Multiple cathepsin-deficient mice were bred from single cathepsin-deficient mice at the University of Massachusetts Medical School. All animal protocols were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Generation of bone marrow chimeras

Adult wild-type (WT) C57BL/6 mice were lethally irradiated (1100 rad) and reconstituted for at least 8 wk with bone marrow collected from age-matched WT or mutant donor mice 1–2 wk old. Some recipient mice in each group expressed the leukocyte marker Ly5.1 (CD45.1), whereas all donors expressed Ly5.2 (CD45.2), allowing confirmation of >90% chimerism to be determined by flow cytometric analysis of peripheral blood samples.

Production and measurement of IL-1β, pro–IL-1β, and TNF-α from in vitro cultures

Peritoneal exudate cells were elicited by i.p. injection of 3 ml 1% thioglycollate and collected after 72 h by peritoneal lavage. Prior to experimentation, non-adherent cells were decanted, leaving primarily macrophages behind. Bone marrow–derived macrophages were generated as described previously (60). Bone marrow neutrophils were isolated from whole bone marrow, following RBC lysis, using the anti–Ly-6G Microbead Kit from Miltenyi Biotec. Purity was assessed to be 95–98% by flow cytometry. Murine bone marrow–derived mast cells were derived from whole bone marrow using murine rIL-3 (PeproTech), and purity was assessed to be 95% by toluidine blue (61). Cells were plated overnight in 96-well plates (ELISA) or 12-well plates (SDS-PAGE, cathepsin activity labeling with BMV109, and Western blotting). Unless otherwise stated, the "standard protocol" followed in this study is as follows: priming in RPMI 1640 medium (or MC/9 medium for mast cells (61)) for 3 h with LPS (200 ng/ml), with or without the addition of inhibitors after 2 h of priming, followed by 6 h of stimulation. Inhibitors were added in a final concentration of ≤0.1% DMSO, which has no effect on readouts compared with medium alone. Supernatants were collected, with or without addition of Promega's 10× lysis solution for measuring intracellular cytokines or lactate dehydrogenase (LDH) measurement by plate reader at OD_{490} using Promega's Cytox96 Nonradioactive cytotoxicity assay, and cytokine levels were analyzed by ELISA.

siRNA knockdowns

Each siRNA was used at a 50 nM final concentration (or control siRNA at 100 nM for double knockdowns) after complexation in a mixture of Endoporter (GeneTools) and RNAiMax (Life Technologies) at a ratio of 0.11 μl:0.15 μl in OptiMEM (Life Technologies), respectively, per 0.1 ml final volume (in 10% FCS). Complexes were combined with 10% FCS-containing RPMI 1640 medium at a ratio 1:10 (complexes:10% FCS) and added to cells for 96 h. Medium was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.2 mM 2-ME, 1× nonessential amino acids, and 100 μg/ml ciprofloxacin.

Immunoblotting and Live-cell intracellular cathepsin activity labeling with BMV109

In 12-well plates, adherent macrophages were washed with RPMI 1640 medium, and incubated with or without LPS as indicated for 3 h (inhibitors

added after 2 h), at which time BMV109 (62) was added at a final concentration of 1 μM. After 1 h with BMV109, supernatants were collected, cells were washed with PBS, and lysates were made with Cell Extraction Buffer from Life Technologies with complete protease inhibitor mixture from Roche. Supernatants were precipitated with chloroform/methanol, and lysate protein concentration was equalized using the Pierce BCA Assay. At least 15 μg was loaded for each sample and separated by 15% (or 8% for NLRP3 blots) SDS-PAGE, and gels were analyzed with a Typhoon Trio phosphor-imager from GE, and protein was transferred onto nitrocellulose membranes. Densitometry was performed using ImageJ. Images of gels or blots were cropped for the bands of interest, and any contrast enhancement was applied evenly throughout using iPhoto.

Real-time measurement of lysosomal integrity

In black high-binding 96-well clear-bottom plates, 50 μl acridine orange (Life Technologies) in warm HBSS (with Ca^{2+} and Mg^{2+}) was added to cells in 100 μl RPMI 1640 medium containing 10% FCS to reach a final concentration of 3.75 μg/ml and then incubated at 37°C for 15 min prior to washing 1× with 200 μl HBSS. Cells were then treated and stimulated as indicated in phenol red-free CO_2-independent Leibovitz's medium. Fluorescence was measured in each well every 1–3 min using an incubated VictorX5 plate reader. Background fluorescence was subtracted from wells treated with dye-free HBSS.

Results

Genetic and biochemical analysis of the impact of individual cathepsin deficiency on particle-induced IL-1β secretion

The role of cathepsins in NLRP3 activation remains controversial. Some studies describe a role for cathepsin B or L (6, 25, 41), whereas others show no role for either cathepsin in particle-induced NLRP3 activation and IL-1β secretion (31, 42, 47). One interpretation of these data suggests that other cathepsins, besides B or L, may be the key players in this response. Therefore, we examined the impact that genetic deficiency of five closely related individual cathepsins has on particle-induced IL-1β secretion. Unless noted otherwise, IL-1β secretion was induced with various stimuli following 3 h of LPS priming. First, we examined peritoneal macrophages (PMs) elicited from mice lacking cathepsins B, L, S, or C. However, these cathepsin-deficient PMs displayed no difference in IL-1β secretion in response to silica compared with PMs derived from WT mice (Fig. 1A). To examine the role of cathepsin X, we silenced cathepsin X in PMs by siRNA knockdown, and then, these cells were stimulated with silica, the soluble NLRP3 activator nigericin, or the Absent in melanoma 2 inflammasome activator poly(deoxyadenylic-deoxythymidylic) acid (dAdT) (Fig. 1B). We confirmed a 90–95% knockdown of cathepsin X mRNA by quantitative PCR (qPCR) (Fig. 1C) and noted a similar loss of cathepsin X activity using the fluorescent activity–based probe BMV109 (Fig. 1D), which binds covalently to active cathepsins inside live cells (62). In lysates generated from these cells, the proteins were separated by SDS-PAGE, and then, the activity of specific cathepsins was assessed in the gels using a laser phosphor-imager to analyze the degree of fluorescence for each cathepsin at the appropriate molecular mass. Again, we noted no significant difference in IL-1β secretion between cathepsin X–sufficient and cathepsin X–deficient PMs in response to either silica or dAdT. Strikingly, cathepsin X deficiency significantly reduced IL-1β secretion in response to nigericin. In contrast, LPS-induced TNF-α secretion was unaffected by the loss of any of the cathepsins tested. Therefore, the individual cathepsins B, L, S, C, and X are dispensable for silica-induced IL-1β secretion, but we found, unexpectedly, that in the response induced by nigericin, cathepsin X plays a nonredundant role.

Using cathepsin knockout animals to study IL-1β secretion could potentially be confounded if some cathepsins are upregulated to compensate for the deficiency of others (54, 63, 64). Using

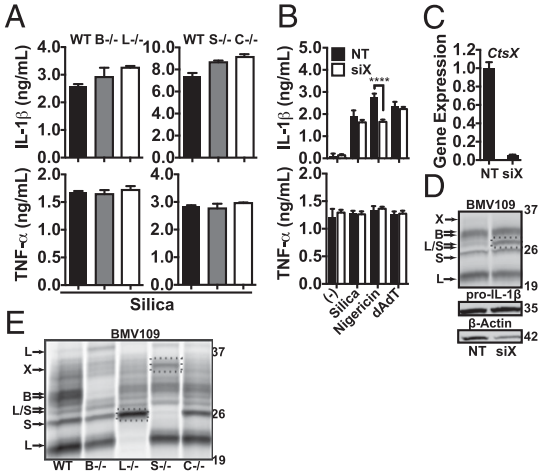

FIGURE 1. Sterile particle-induced IL-1β secretion does not require cathepsins B, L, C, S, or X, but nigericin is partially dependent on cathepsin X. (**A**) LPS-primed PMs from WT mice or mice deficient for cathepsins B ($B^{-/-}$), L ($L^{-/-}$), S ($S^{-/-}$), or C ($C^{-/-}$) were stimulated with silica (40 μg/ml). (**B**) PMs were treated with nontargeting (NT) control siRNA or siRNA targeting cathepsin X (siX) before priming with LPS and stimulating with media control (−), silica (80 μg/ml), nigericin (1.5 μM), or dAdT (0.5 μg/ml). IL-1β and TNF-α were measured in supernatants by ELISA. (**C**) PMs from "B" were analyzed for cathepsin X (*CtsX*) expression by qPCR following siRNA (siX) treatment and LPS priming; data are normalized to GAPDH expression and plotted relative to NT siRNA, or (**D**) cathepsin X activity was probed with BMV109; lysates were processed and pro–IL-1β and β-actin analyzed by Western blot; dashed box highlights upregulated cathepsin L/S activity; molecular mass markers are on the right in kilodaltons. (**E**) LPS-primed PMs from WT or cathepsin-deficient mice in (A) were probed for cathepsin activity with BMV109; dashed boxes highlight upregulated cathepsin S or X activity in $L^{-/-}$ and $S^{-/-}$ macrophages; molecular mass markers are on the right in kilodaltons. Error bars represent range bars of technical duplicates (A), SD of technical quadruplicates (B), and SD of technical triplicates (C). (B) All data are representative of at least three independent experiments. Statistical analysis was performed by two-way ANOVA and Sidak's multiple comparisons test, $****p < 0.0001$.

BMV109, we examined the activity of specific intracellular cathepsins in the LPS-primed WT and cathepsin-deficient PMs that were tested above in Fig. 1A and 1B. Indeed, knockdown of cathepsin X with siRNA resulted in an upregulation of cathepsin L and S activity (Fig. 1D). Moreover, PMs lacking cathepsin L showed increased cathepsin S activity, whereas those deficient in cathepsin S upregulated cathepsin X activity (Fig. 1E). Taken together, these data indicate that the cathepsins examined, including cathepsins B, L, S, and X, are not essential for particle-induced IL-1β secretion, and they cannot be readily studied using genetic methods because of compensation issues upon knockdown.

Analysis of small-molecule cathepsin inhibitors

The absence of a phenotype in cathepsin B–deficient macrophages, shown in this paper and reported by others, contradicts the results reported with cathepsin B inhibitors (31, 42, 47). Despite several reports demonstrating that the cathepsin inhibitor Ca074Me inhibits multiple cathepsins in biochemical and cellular assays (48–52), Ca074Me is cited as a cathepsin B–specific inhibitor and used to implicate cathepsin B in NLRP3 activation in many studies (2, 7, 17, 20, 21, 25–29, 41–46). The nonselective prodrug methyl ester Ca074Me is processed in lysosomes into the highly cathepsin B–selective free acid Ca-074. However, this processing occurs slowly and allows time for Ca074Me to inhibit multiple

cathepsins (48–52). Therefore, in the context of NLRP3 activation, Ca074Me's targets in intact cells have not yet been verified and closely examined as a function of inhibitor concentration. In this study, we re-examine both Ca074Me and a newly described broad cathepsin inhibitor, K777 (*N*-methyl-piperazine-phenylalanyl-homophenylalanyl-vinylsulfone-phenyl), whose anti-inflammatory properties have not yet been tested. K777 inhibits cathepsins B, L, S, C, V, and K in cell-free assays (65). Using Ca074Me or K777 in combination with the active site probe allowed us to correlate their effects on IL-1β secretion with the extent of inhibition of specific cathepsins as a function of concentration.

To examine the inhibition profile of K777, we treated PMs with K777 or solvent control (DMSO) for 1 h (after 2 h of LPS priming, unless stated otherwise), after which we probed for cathepsin activity in the intact cells with BMV109. As previously reported, K777 inhibited cathepsins B, L, and S (65) over a titration range from 0.1 to 30 μM (Fig. 2A, 2B). Interestingly, we also found that K777 inhibited cathepsin X at high concentrations but unexpectedly increased cathepsin X activity at lower concentrations. These paradoxical effects can be explained by K777's greater potency toward cathepsin S, which fits with our data, in Fig. 1E above, showing that cathepsin S deletion causes an increase in cathepsin X activity. Therefore, K777 inhibits cathepsin S at low concentrations, which likely causes a compensatory increase in cathepsin X activity.

In parallel to examining its effects on cathepsin activity, we also tested the effect of K777 on IL-1β secretion (Fig. 2C). PMs were primed with LPS and treated with K777 as done above (2 h after LPS priming and 1 h prior to stimulation), at which point they were exposed to various stimuli for an additional 6 h of incubation; this is the standard protocol used for the rest of this study, unless stated otherwise. At concentrations where multiple cathepsins were inhibited, K777 suppressed silica-induced IL-1β secretion. In contrast to silica, K777 was much less effective at suppressing IL-1β secretion induced by nigericin. Presumably, this is because K777 has opposing effects on cathepsin X, which is uniquely required for the nigericin response, shown in Fig. 1B. Moreover, K777 had a negligible affect on the IL-1β response induced by dAdT. We also confirmed that K777 is similarly selective and/or efficacious at suppressing IL-1β secretion induced by other particles, including alum and CC, and in other primary myeloid cell lines, including bone marrow–derived macrophages, mast cells, and neutrophils (Supplemental Fig. 1A, 1B). Importantly, K777 did not affect LPS-induced TNF-α production within the tested concentration range, suggesting specific inhibition of IL-1β secretion.

We performed similar analyses for Ca074Me (Fig. 2D–F). Although Ca074Me was selective for cathepsin B at concentrations < 1 μM, at higher concentrations (typically used in previous studies) it inhibited cathepsins broadly (Fig. 2D, 2E). Moreover, >10 μM Ca074Me was required to completely inhibit cathepsin B. Unlike K777, Ca074Me suppressed nigericin and silica-induced IL-1β secretion with similar potency, presumably because Ca074Me inhibits cathepsin X more potently than K777 (Fig. 2F). Interestingly, the concentration required to achieve and maximize these effects exceeds the range in which Ca074Me is selective for cathepsin B. In reviewing previous studies examining

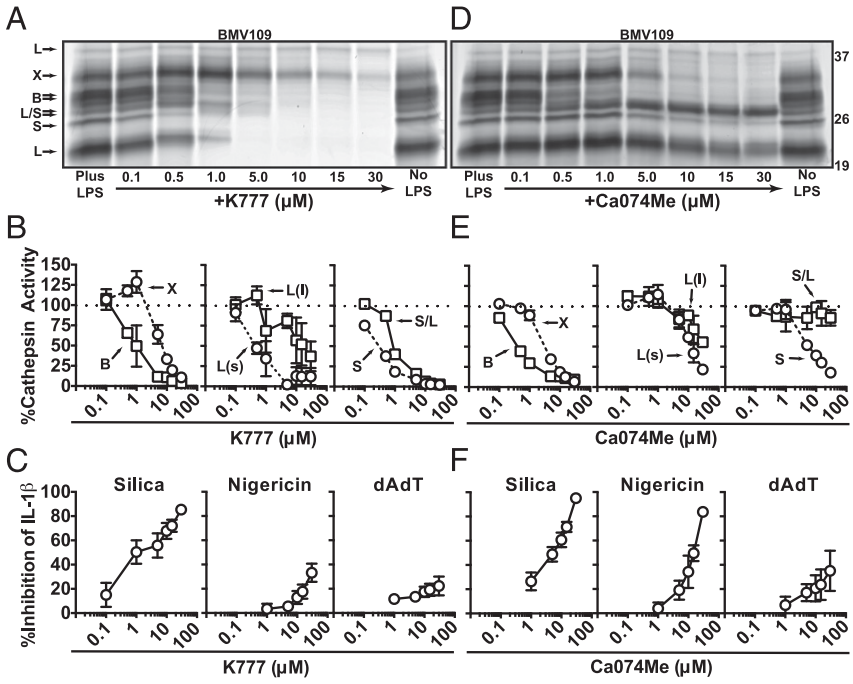

FIGURE 2. Both Ca074Me and K777 inhibit multiple cathepsins at concentrations needed to block IL-1β secretion. (**A**) PMs were given media control (No LPS) or LPS-primed (Plus LPS; +K777) and subsequently treated with media control (No LPS; Plus LPS) or the indicated concentrations of K777 (+K777), after which cathepsin activity was labeled with BMV109 in live cells before lysates were processed by SDS-PAGE and phosphor imaged; molecular mass markers are on the right in kilodaltons. (**B**) Concentration-dependent inhibition of cathepsin activity by K777 analyzed by densitometry of (A): cathepsin B (□) and X (○), large ($L_{(l)}$; □) and small ($L_{(s)}$; ○) molecular mass isoforms of cathepsin L, cathepsin S (○), and overlapping molecular mass isoforms of S and L (S/L; □). (**C**) LPS-primed PMs were treated with media control or the indicated concentrations of K777 and stimulated with silica (40 μg/ml), nigericin (2 μM), or dAdT (0.5 μg/ml); data show percent inhibition of IL-1β secretion measured in supernatants compared with no inhibitor treatment. (**D**–**F**) Same as (A–C) but with Ca074Me instead of K777. Error bars represent SE of means from three independent experiments (B), SD of means from four independent experiments (0.1–15 μM) or S.D. of means from three independent experiments (30 μM) (C), range bars of the means from two independent experiments (E), or SD of the means from three independent experiments (0.5–15 μM) (F), or range bars of the means from two independent experiments (30 μM).

Ca074Me's effects on IL-1β responses, the concentrations used were also in the range that would inhibit multiple cathepsins (10–200 μM) (2, 7, 17, 20, 21, 25–29, 41–46). Therefore, our findings likely explain the difference in results seen for the genetic loss of cathepsin B compared with small-molecule inhibitors of this protease. In summary, although both K777 and Ca074Me inhibit multiple cathepsins at concentrations required to suppress IL-1β secretion, K777 blocks particle-induced NLRP3 activation more selectively than Ca074Me.

To further investigate whether Ca074Me or K777 can inhibit IL-1β secretion in PMs from cathepsin-deficient mice, these cells were LPS primed and treated with inhibitors prior to stimulation. Indeed, K777 inhibited IL-1β secretion to the same extent in WT cells as in cathepsins B, L, S, or C–deficient cells (Fig. 3A). Moreover, across a titration range for both K777 and Ca074Me, the extent to which they suppressed IL-1β secretion was the same in both WT and cathepsin B–deficient PMs (Fig. 3B). Again, LPS-induced TNF-α secretion was relatively unaffected by cathepsin B deficiency or inhibitor treatments. Taken together, these data indicate that the individual cathepsins examined, including cathepsin B, are not essential for the activation of particle-induced IL-1β secretion or as targets for cathepsin inhibitors that suppress this response.

Analysis of compound cathepsin deficiencies

The analyses above suggest that multiple cathepsins likely play compensatory roles in particle-induced IL-1β secretion. This is in line with some genetic evidence that has shown partial or conditional involvement for cathepsin B or L in NLRP3 activation (6, 25, 41). In fact, these two cathepsins have been shown to compensate for one another in a study demonstrating that combined cathepsin B and L deficiency is neonatal lethal in mice, but deficiency of either protease alone is nonlethal (54). Therefore, a dual deficiency of cathepsins B and L may have a greater effect on the IL-1β response (6).

To test this hypothesis, we bred mice lacking both cathepsins B and L. Because combined cathepsin B and L deficiency is neonatal lethal (54), we could not analyze responses directly in these animals. Instead, we harvested bone marrow from neonates and used it to reconstitute lethally irradiated adult WT mice. In these chimeric mice, cells of hematopoietic origin lack cathepsin B and L (B&L$^{-/-}$). For comparison, we made similar chimeras with WT, cathepsin B$^{-/-}$, and cathepsin L$^{-/-}$ bone marrow. Then, we elicited PMs from these chimeric mice and treated them as above. We verified that the PMs collected from these chimeric mice lacked activity for the appropriate cathepsins using BMV109 (Supplemental Fig. 2A). Again, we observed upregulation of cathepsin S activity upon loss of cathepsin L. However, cathepsin B$^{-/-}$, L$^{-/-}$, or B&L$^{-/-}$ PMs showed no attenuation of IL-1β secretion in response to the lysosome-disrupting agent Leu-Leu-OMe (LLOMe), silica, nigericin, or dAdT (Fig. 4A). Moreover, there was no defect in IL-1β secretion over a broad titration of silica (Fig. 4B). Interestingly, K777 still suppressed silica-induced IL-1β secretion in the absence of cathepsins B and/or L (Fig. 4C), suggesting that other cathepsins potentially contribute to this response.

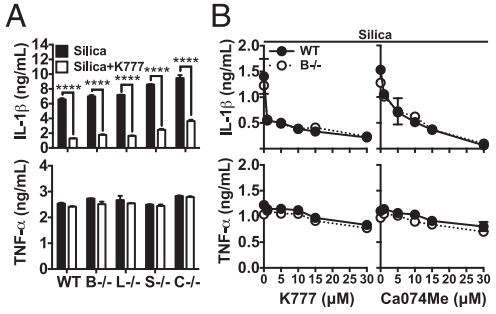

FIGURE 3. Cathepsin inhibitors suppress particle-induced IL-1β secretion independently of individual cathepsins. IL-1β (*upper graphs*) and TNF-α (*lower graphs*) were measured in supernatants. (**A**) LPS-primed WT PMs or those lacking cathepsins B (B$^{-/-}$), L (L$^{-/-}$), S (S$^{-/-}$), or (C$^{-/-}$) were treated with silica (■; 40 μg/ml) or silica plus K777 (□; 15 μM). (**B**) LPS-primed WT (●, solid line) or cathepsin B–deficient (○, dashed line) PMs were treated silica (50 μg/ml) or silica plus a range of K777 or Ca074Me concentrations (1, 5, 10, 15, or 30 μM). Error bars represent range bars of technical duplicates. Statistical analysis was performed by two-way ANOVA and Sidak's multiple comparisons test, ****$p < 0.0001$. Data are representative of two [(B) for Ca074Me] or three [(A and B) for K777] independent experiments.

Because both K777 and Ca074Me inhibit more cathepsins than just B and L at the concentrations required to block particle-induced IL-1β secretion, we examined the particle-induced responses of macrophages genetically deficient in up to five cathepsins (Fig. 4D, 4E). To do this, we elicited PMs from WT mice or mice deficient in the three cathepsins B, C, and S (BCS$^{-/-}$), which are viable with no obvious physical or behavioral pathology. In addition, in both WT and BCS$^{-/-}$ macrophages, we silenced cathepsins X and L with siRNA (siXL; XL) or treated cells with nontargeting siRNA (WT). This resulted in a 90–95% reduction in mRNA of each targeted gene and reduction in enzyme activity, as assayed with BMV109 (Supplemental Fig. 2B, 2C). Finally, PMs were primed with LPS, with medium or K777 treatment, and stimulated with silica, nigericin, or dAdT as done above. Indeed, macrophages deficient in the five cathepsins B, C, S, X, and L (BCSXL) showed a significant, although small, reduction in IL-1β secretion in response to silica but not nigericin or dAdT (Fig. 4D). However, K777 was still effective at further suppressing IL-1β secretion in these macrophages. Interestingly, in the lysates of samples treated with LPS only, we observed a similar decrease in intracellular IL-1β levels, suggesting that lower levels of IL-1β synthesis may be contributing to the reduction in IL-1β secretion seen for both BCSXL deficiency and K777 treatment (Fig. 4E). Again, we observed a compensatory upregulation of cathepsin activity, with cathepsin B and S activity upregulated in the cathepsin XL knockdown and increased cathepsin X activity in the cathepsin BCS$^{-/-}$ PMs (Supplemental Fig. 2C). This may explain why nigericin was not significantly affected by knockdown of cathepsin X in combination with these other cathepsin deficiencies. As above, TNF-α secretion was unaffected, suggesting that compound cathepsin deficiency specifically impacts the IL-1β pathway (Supplemental Fig. 2D–H). Thus, compound deficiency of cathepsins B, C, S, X, and L demonstrates a reproducible, albeit minor, attenuation of particle-induced IL-1β secretion. However, the fact that cathepsin inhibitors have shown, yet again, more profound effects on IL-1β secretion than that caused by genetic deficiency suggests that additional cathepsins or possibly other targets affected by the inhibitors might be involved in particle-induced IL-1β secretion.

Analysis of endogenous cathepsin inhibitors

Although technical limitations prevent us from genetically deleting all potentially relevant cathepsin activity, these proteases are specifically inhibited by a family of endogenous regulators called cystatins (66). Therefore, we examined the effects of genetically disabling the activity of the endogenous cathepsins inhibitors, cystatin C and B, on particle-induced IL-1β secretion.

We used siRNA to silence cystatin C, B, or C and B in PMs to investigate their role in IL-1β secretion (Fig. 5). In all cases, we

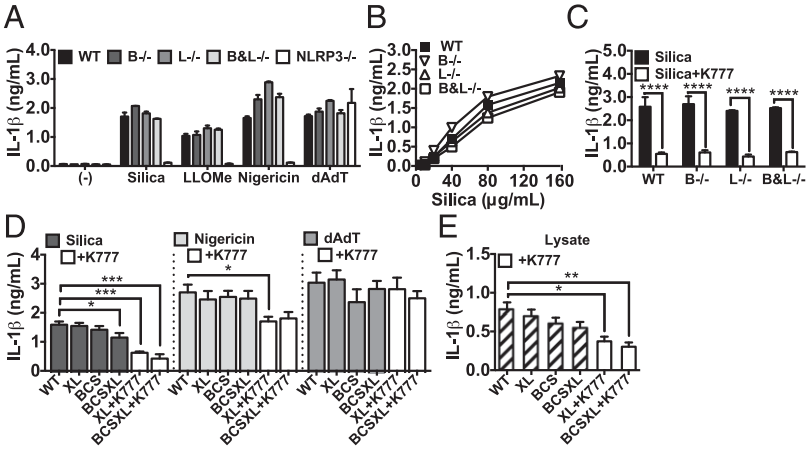

FIGURE 4. Compound cathepsin deficiency causes a minor reduction in particle-induced IL-1β secretion. IL-1β was measured in supernatants. (**A**–**C**) Lethally irradiated WT mice were reconstituted with bone marrow from WT, cathepsin B ($B^{-/-}$), L ($^{-/-}$), B, and L ($B\&L^{-/-}$), or NLRP3 ($NLRP3^{-/-}$)–deficient donor mice. LPS-primed PMs elicited from these mice were stimulated with media control (−), silica (40 μg/ml), LLOMe (0.75 mM), nigericin (2 μM), or dAdT (0.4 μg/ml) (A), a range of silica concentrations (B), and silica plus media (■) or silica plus K777 (□; 20 μM) (C). (**D**) PMs elicited from WT or mice deficient in the three cathepsins B, C, and S (BCS) were treated with nontargeting siRNA (WT) or siRNA targeting both cathepsins X and L ("XL" when given to WT, or "BCSXL" when given to BCS) and subsequently LPS-primed and stimulated with media control (−), silica (80 μg/ml), nigericin (1.5 μM), or dAdT (0.5 μg/ml). XL and BCSXL macrophages were also treated with K777 (XL+K777 and BCSXL+K777; □; 15 μM). Error bars represent range bars of technical duplicates (A–C) or SE of means (D) from either five independent experiments (WT, XL, BCS, and BCSXL) or three independent experiments (+K777). All data are representative of at least three independent experiments. Statistical analysis was performed by Two-way ANOVA and Sidak's multiple comparisons test (A–C) or two-tailed Student *t* test (D and **E**): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

achieved ∼95% knockdown of cystatin expression (Supplemental Fig. 3A). Indeed, cystatin C deficiency alone caused a significant increase in silica and nigericin-induced IL-1β secretion but not following stimulation with dAdT (Fig. 5A). Moreover, combined deficiency of cystatin B and C synergistically enhanced IL-1β secretion for all stimuli tested. In the absence of these cystatins, K777 selectively reduced silica-induced IL-1β secretion. Therefore, cystatins C and B appear to nonspecifically regulate the level of IL-1β secretion, whereas cystatin C preferentially affects particulate and NLRP3-activating stimuli.

Surprisingly, knockdown of cystatin C and/or B caused an upregulation of pro–IL-1β transcript levels induced by LPS priming and an increase in the level of mature IL-1β and pro–IL-1β detected in lysates; mature IL-1β detected in lysates by ELISA after LPS priming directly reflects levels of pro–IL-1β (Fig. 5B–E). Although this effect is more prominent with cystatin C deficiency, knockdown of both cystatin C and B synergistically enhances pro–IL-1β synthesis. Assessment of cellularity by detergent-induced LDH release (OD_{490}) indicated that the elevation in pro–IL-1β levels was not a result of enhanced proliferation during knockdown (Supplemental Fig. 3B). Interestingly, the observed elevation in pro–IL-1β synthesis was proportional to increases observed in IL-1β secretion following stimulation with silica, nigericin, or dAdT. Moreover, K777 suppressed the increase in pro–IL-1β synthesis and IL-1β secretion resulting from cystatin C and B knockdown, specifically for silica. The fact that K777 reduced pro–IL-1β synthesis more effectively than it reduced IL-1β secretion induced by nigericin and dAdT may reflect that intracellular levels of pro–IL-1β were not limiting for these stimuli and/or that there are kinetic differences in pro–IL-1β induction with the different stimuli. Alternatively, cathepsins may also play a selective role in particle-induced NLRP3 activation (Signal 2) as originally proposed.

Given that cathepsins are not known to play a role in Signal 1 (LPS priming), our finding that cystatins regulate pro–IL-1β synthesis is surprising. However, this is consistent with our observation that the multiply-deficient BCSXL PMs have a lower level of IL-1β detected in the lysate that seems proportional to the reduction in IL-1β secretion. In fact, this indicates that previous findings of lower IL-1β secretion from cathepsin-deficient macrophages may be a direct result of depressed pro–IL-1β synthesis. Indeed, careful examination revealed that cathepsin $B\&L^{-/-}$ or $BCS^{-/-}$ macrophages have partial but significant reductions in intracellular IL-1β/pro–IL-1β detected in lysates after LPS priming by either ELISA or Western blot (Fig. 5F, 5G). The fact that we did not see a significant reduction in secreted IL-1β corresponding to the reduction in intracellular IL-1β/pro–IL-1β is presumably because the reduced pro–IL-1β levels were not below the threshold required to limit the response. Importantly, no single-cathepsin deficiency significantly reduced intracellular IL-1β levels (Supplemental Fig. 3C, 3D). Therefore, this effect was not responsible for the reduction in the response to nigericin after silencing cathepsin X (Fig. 1D, Supplemental Fig. 3D). In any case, our data indicate that cathepsins do indeed play a role in pro–IL-1β synthesis. Notably, LPS-induced TNF-α secretion is relatively unaffected, suggesting that the impact of cystatin deficiency or K777 treatment on pro–IL-1β synthesis does not apply to all NF-κB–dependent cytokines (Supplemental Fig. 3E). Also, knockdown of cystatins B and C also enhanced NLRP3 transcript levels and NLRP3 protein synthesis but to a lesser extent than for IL-1β (Supplemental Fig. 3F, 3G). Our data indicate a previously unreported and significant role for cathepsins and their endogenous inhibitors in pro–IL-1β synthesis and that cystatins B and C regulate particle-induced IL-1β secretion by suppressing multiple cathepsins involved in mediating pro–IL-1β synthesis.

Analyzing the effect of small molecule cathepsin inhibitors on pro–IL-1β synthesis

We demonstrated that cathepsin deficiency attenuates pro–IL-1β synthesis, whereas cathepsin deregulation by cystatin C and B knockdown enhances pro–IL-1β synthesis. These data indicate that cathepsin inhibitors may suppress IL-1β secretion by affecting pro–IL-1β synthesis. However, if this is true, it is surprising that K777 and Ca074Me did not similarly suppress dAdT-induced IL-1β secretion in previous experiments. However, the kinetics of LPS priming is an important variable when considering the effect

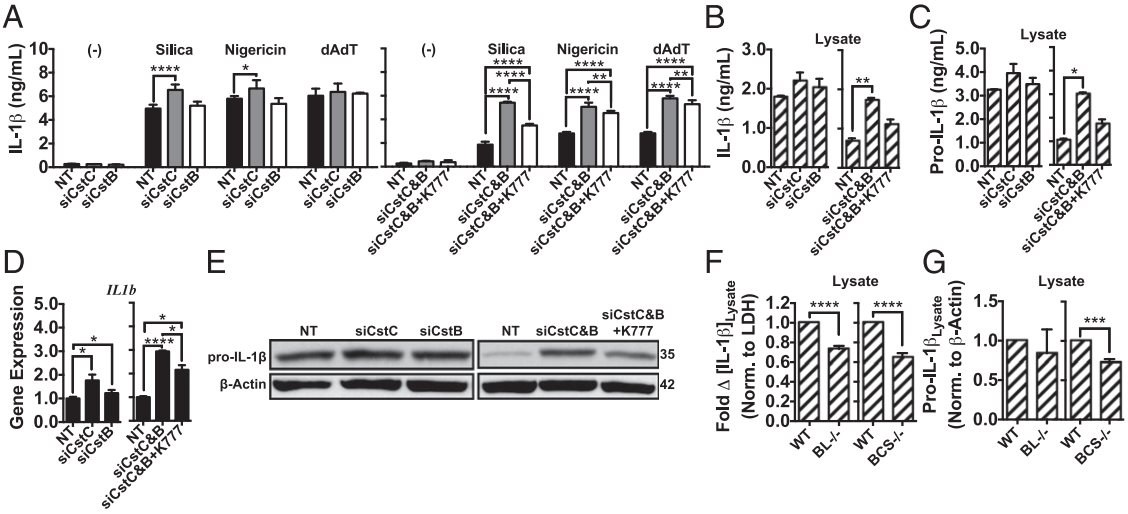

FIGURE 5. Endogenous cathepsin inhibition by cystatins C and B regulates particle-induced IL-1β secretion and LPS-induced pro–IL-1β synthesis. In all experiments, PMs were LPS-primed and treated with media control or K777 (+K777; □; 15 μM) prior to stimulation or analysis. (**A**–**E**) PMs were transfected with nontargeting (NT), cystatin C (siCstC), cystatin B (siCstB), or both cystatin C and B (siCstC&B) siRNA. (A) PMs were stimulated with media control (−), silica (80 μg/ml), nigericin (1.5 μM) or dAdT (0.5 μg/ml), and IL-1β measured in supernatants. (B–E) After priming, PMs were treated with media control for 6 h. IL-1β (B) or pro–IL-1β (C) was measured in cell lysates by ELISA. (D) IL-1β (*IL1b*) expression was analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to NT siRNA. (E) Lysates were processed, and then, pro–IL-1β and β-actin analyzed by Western blot; molecular mass markers are on the right in kilodaltons. (**F** and **G**) PMs from WT mice and cathepsin BCS$^{-/-}$ mice or chimeric WT mice lethally irradiated and reconstituted with WT or cathepsin BL$^{-/-}$ bone marrow. PMs were treated with media for 6 h after LPS priming. (F) IL-1β (hatched bars) was measured in lysates by ELISA; data are normalized to LDH (OD_{490}) and plotted as fold change in IL-1β relative to WT controls. (G) Lysates were processed and analyzed for pro–IL-1β and β-actin by Western blot (measured by densitometry); data are plotted as pro–IL-1β levels normalized to β-actin and relative to WT controls. Error bars represent SD of technical quadruplicates (A), range bars of technical duplicates (B and C), SD of technical triplicates (D), SE of means from 9 (WT versus BCS$^{-/-}$) or 12 (WT versus BL$^{-/-}$) independent experiments (F), SE of means from 5 (WT versus BCS$^{-/-}$) or 4 (WT versus BL$^{-/-}$) independent experiments (G). All data are representative of at least three independent experiments. Statistical analysis was performed by two-way ANOVA and Dunnett's multiple comparisons test (A), one-way ANOVA and Sidak's multiple comparisons test (B–D), or two-tailed Student *t* test (F and G): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

of inhibitors on IL-1β secretion, and influences on priming seem to be selective for NLRP3-dependent stimuli compared with those activating other inflammasomes (15). Therefore, we examined whether cathepsin inhibitors affect pro–IL-1β synthesis and how the timing of inhibitor treatment affects their specificity.

To test the effect of cathepsin inhibitors on pro–IL-1β synthesis, we varied the timing of inhibitor treatment relative to LPS priming using an "early versus late inhibitor treatment protocol" (Fig. 6A–C). First, we treated PMs with K777, Ca074Me, or the pan-caspase inhibitor ZVAD immediately prior to LPS priming. In a parallel sample set, we added these inhibitors just prior to stimulation, 3 h after LPS priming, and examined how treatment with inhibitors at this time point compares with the former. K777 or Ca074Me treatment prior to LPS priming suppressed both pro–IL-1β in macrophage lysates (Fig. 6A) and IL-1β secretion by silica, nigericin, and dAdT (Fig. 6B). Moreover, these effects were greater for inhibitor treatment just prior to priming. K777 or Ca074Me treatment 3 h after LPS priming (just before stimulation) had no effect on dAdT, and as shown earlier, Ca074Me had a more potent effect on nigericin-induced IL-1β secretion (Fig. 6B).

To determine whether the reductions in IL-1β secretion that we previously observed were also a reflection of reduced pro–IL-1β levels, we retested K777 and Ca074Me using the standard protocol described for these earlier experiments and examined their effects on pro–IL-1β synthesis (Fig. 6C–E). Indeed, treatment with K777 or Ca074Me after only 2 h of LPS priming reduced pro–IL-1β levels in lysates (Fig. 6D) and also reduced pro–IL-1β transcript levels (Fig. 6E). In fact, K777 even suppressed NLRP3 transcript levels, although the reduction in NLRP3 transcript caused by Ca074Me was not significant. In contrast to the near complete inhibition of IL-1β secretion by all stimuli, ZVAD treatment had no effect on intracellular IL-1β or pro–IL-1β levels detected in LPS-primed macrophage lysates (Fig. 6, Supplemental Fig. 4A). Moreover, ZVAD did not suppress pro–IL-1β and NLRP3 transcript levels or cathepsin activity. Again, under all these conditions above, TNF-α secretion remained unaffected (Supplemental Fig. 4B). Therefore, cathepsin inhibitors suppressed the synthesis of pro–IL-1β and not TNF-α. When added just prior to LPS priming, cathepsin inhibitors also attenuated NLRP3-independent IL-1β secretion, yet they maintained some selectivity for NLRP3-dependent IL-1β secretion (Fig. 6B). These findings are consistent with a previous study showing that several inhibitors, which also affect Signal 1, preferentially affect NLRP3-dependent stimuli (15). Indeed, the persistent selectivity of cathepsin inhibitors for NLRP3-dependent stimuli may reflect a unique dependence of these responses on Signal 1, based on the requirement for de novo NLRP3 transcription or some other factor yet to be defined. Although this is less likely a reflection of differences in Signal 2 kinetics, which are similar for silica and dAdT (Supplemental Fig. 4C, 4D), the ultimate reason for this difference is unclear because the effects of K777 and Ca074Me on pro–IL-1β protein levels are more pronounced than their effects on NLRP3 protein levels (Supplemental Fig. 4E).

Analyzing the effect of cathepsin inhibitors on Signal 2 of NLRP3 activation

We found that cathepsin inhibition by both small molecules and endogenous regulators suppresses pro–IL-1β synthesis. However, we expected that these effects on pro–IL-1β synthesis would affect all stimuli equally. Instead, cathepsin inhibition had a greater

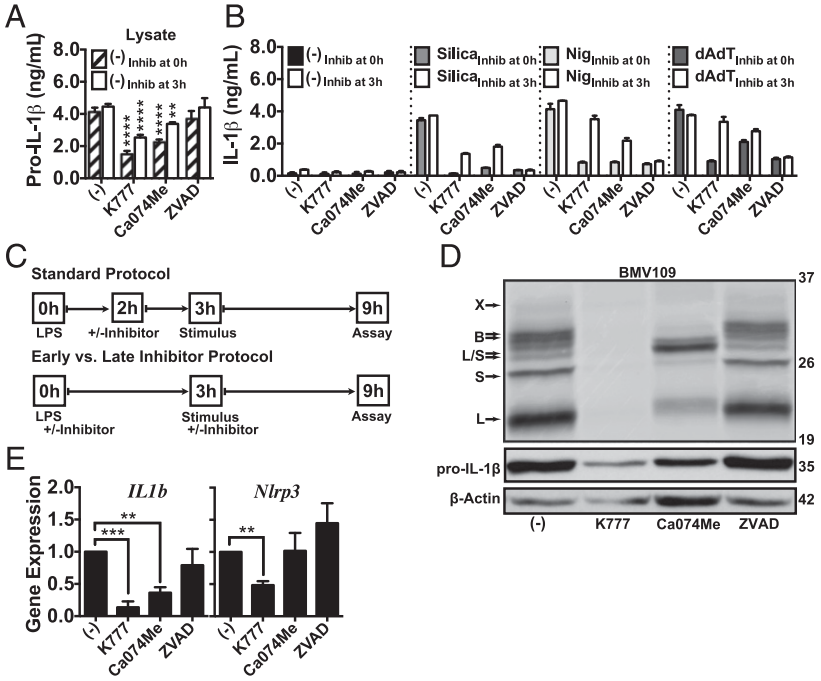

FIGURE 6. Small-molecule cathepsin inhibitors suppress pro–IL-1β synthesis. In all experiments, PMs were primed with LPS for 3 h and then treated with media control (−), K777 (15 μM), Ca074Me (15 μM), or ZVAD (10 μM) at the indicated time points. (**A** and **B**) Inhibitors were added at the same time as LPS (Inhib at 0 h; hatched or filled bars) or 3 h after LPS (Inhib at 3 h; white bars) prior to the addition of media control (−), silica (80 μg/ml), nigericin (1.5 μM), or dAdT (0.5 μg/ml) for an additional 6 h, at which point pro–IL-1β was measured in lysates (A), or IL-1β was measured in supernatants by ELISA (B). (**C**) Comparison of the inhibitor protocol followed in prior figures and in (**D**) and (**E**) (standard protocol) with the protocol used in (A) and (B) (early versus late inhibitor protocol). (D and E) Inhibitors were added 2 h after LPS priming for 1 h, as in the standard protocol, and then, cells were treated with media for 4 h. (D) Cathepsin activity was probed with BMV109 in live cells; lysates were processed by SDS-PAGE and phosphor imaged, or analyzed for pro–IL-1β and β-actin by western blot; molecular mass markers are on the right in kilodaltons. (E) IL-1β (*IL1b*) or NLRP3 (*Nlrp3*) expression was analyzed by qPCR; data are normalized to GAPDH expression and plotted relative to media controls (−). Error bars represent SD of technical triplicates (-) (A and B), duplicates (silica, nigericin, and dAdT) (B), and SE of means from three independent experiments (E). All data are representative of at least three independent experiments. Statistical analysis was performed by two-way ANOVA and Dunnett's multiple comparisons test (A) or two-tailed Student *t* test (E): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

impact on silica-induced IL-1β secretion compared with nigericin or dAdT. Moreover, this selectivity cannot be completely explained by kinetics. Therefore, it was important to determine whether cathepsin inhibitors suppress IL-1β secretion by blocking NLRP3 activation, independently of their effects on pro–IL-1β synthesis.

To determine whether cathepsin inhibition blocks NLRP3-dependent IL-1β secretion (Signal 2) independently of suppressing pro–IL-1β synthesis, we examined the effect of K777 or Ca074Me treatment on IL-1β responses in macrophages with a pool of preexisting pro–IL-1β (Fig. 7A–C). Following an extended priming protocol, we primed PMs with LPS for 5.5 h to build up an intracellular pool of pro–IL-1β, at which time we added K777, Ca074Me, cycloheximide (CHX), or CHX combined with K777 or Ca074Me, and stimulated 30 min later with silica, nigericin, or dAdT for an additional 3 h; CHX blocked new IL-1β synthesis so that we could isolate and analyze the effect of the protease inhibitors on the processing of pro–IL-1β. K777 and Ca074Me had minimal effect on IL-1β or pro–IL-1β protein levels in LPS-primed macrophage lysates at this late time point and also had no additional effect when combined with CHX compared with CHX alone (Fig. 7A, Supplemental Fig. 4F). Importantly, K777 and Ca074Me still attenuated silica-mediated IL-1β secretion, both alone and in the presence of CHX, whereas only Ca074Me affected nigericin-induced activation of the pathway. Again, neither K777 nor Ca074Me blocked dAdT-induced IL-1β secretion, and TNF-α secretion was unaffected (Fig. 7B, Supplemental Fig. 4G).

To determine whether K777 selectively attenuates particle-induced NLRP3 activation, we examined caspase-1 cleavage in response to silica, CC, nigericin or dAdT (Fig. 7C, 7D). Following our standard protocol, we treated PMs with media or K777, 2 h after LPS priming and 1 h prior to stimulation with silica, CC, nigericin or dAdT (Fig. 7c). After 6 h of stimulation, we examined caspase-1 cleavage by Western blot analysis. Interestingly, although K777 reduced pro–IL-1β levels in lysates of LPS-primed macrophages, K777 also suppressed caspase-1 activation and mature IL-1β secretion only after stimulation with silica or CC and not with nigericin or dAdT (Fig. 7D). Importantly, K777 and Ca074Me did not prevent particle-induced lysosomal disruption (Supplemental Fig. 4H). K777 and Ca074Me did suppress LLOMe-dependent lysosomal disruption (Supplemental Fig. 4I), but this was most likely a result of inhibiting of cathepsin C (a known target of K777) activity required for the activation of LLOMe's membrane disruptive properties in the lysosome (65, 67, 68). Therefore, in addition to suppression of pro–IL-1β synthesis, both K777 and Ca074Me can also independently suppress NLRP3 activation, whereas K777 does so selectively for particles without blocking lysosomal disruption.

Taken together, our data suggest a hitherto unrecognized role for cathepsins in inflammasome-mediated IL-1β responses to sterile particles. Furthermore, our study implicates a complex role for cathepsins and their endogenous regulators, cystatins, in regulating not only IL-1β secretion but also IL-1β induction. This highlights the potential for a multistep involvement of this family of proteases during particle-induced inflammation.

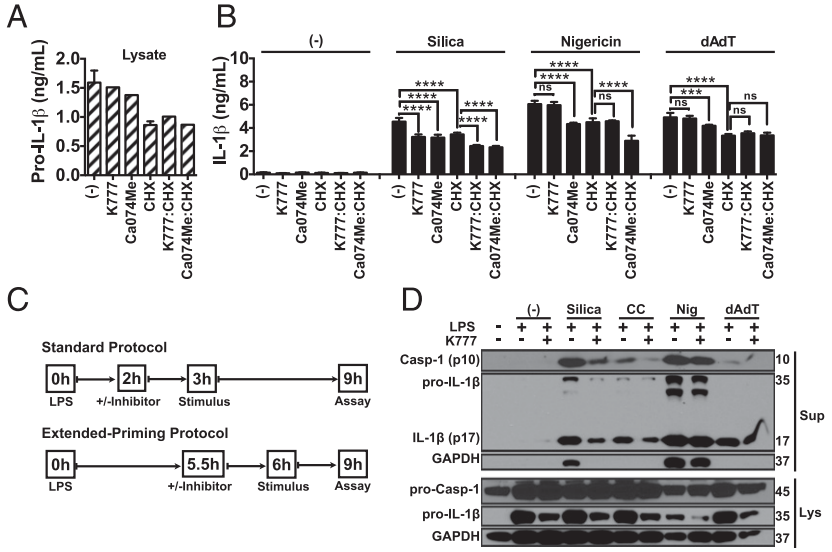

FIGURE 7. Cathepsin inhibitors also suppress NLRP3 activation independently of effects on pro–IL-1β synthesis. (**A** and **B**) PMs were primed with LPS for 5.5 h and treated with either media control (−), K777 (15 μM), Ca074Me (15 μM), CHX (1 μM), K777 combined with CHX, or Ca074Me combined with CHX for another 0.5 h and then treated with media control (−), silica (80 μg/ml), nigericin (1.5 μM), or dAdT (0.5 μg/ml) for another 3 h. Pro–IL-1β (hatched bars) was measured in lysates (A), or IL-1β (filled bars) was measured in supernatants by ELISA (B). (**C**) Comparison of the inhibitor protocol followed in prior figures (standard protocol) with the protocol used in (A) and (B) (extended-priming protocol). (**D**) PMs were either unprimed or primed with LPS and treated with K777 (20 μM) 2 h after LPS priming, as in the standard protocol, and cells were treated 1 h later with media control (−), silica (40 μg/ml), CC (100 μg/ml), nigericin (2 μM), or dAdT (0.4 μg/ml) for an additional 6 h, and then, lysates were processed by SDS-PAGE and analyzed for procaspase-1, active caspase-1 (p-10), pro–IL-1β, active IL-1β (p-17), and GAPDH by Western blot; molecular mass markers are on the right in kilodaltons. Error bars represent SD of technical triplicates (A) and SD of technical triplicates (media or CHX), duplicates (K777 & Ca074Me ± CHX), sextuplicates (silica, nigericin, and dAdT ± CHX), or triplicates (silica, nigericin, and dAdT with K777 and Ca074Me ± CHX) (B). Data are representative of two (A and B) or at least three (D) independent experiments. Statistical analysis was performed by two-way ANOVA and Dunnett's multiple comparisons test (B): $***p < 0.001$, $****p < 0.0001$.

Discussion

Cathepsin B has been implicated in the activation of NLRP3 inflammasomes by particulate stimuli. In this report, we show that, contrary to earlier suggestions, multiple cathepsins are involved redundantly in the production of IL-1β induced by sterile particles. These data address and potentially reconcile earlier controversies on the role of cathepsins. Surprisingly, our data are consistent with a role for cathepsins not only in the NLRP3-dependent maturation of pro–IL-1β but also suggest that they play a substantial role in the priming phase of this response.

Given the controversial role of cathepsins in NLRP3-dependent IL-1β responses (2, 7, 17, 20, 21, 25–29, 31, 41–47), it was important to clarify their contribution by performing a rigorous analysis of two confounding variables that have likely influenced prior results and caused confusion. First, we found that the loss of certain cathepsins causes a compensatory upregulation in the activity of other cathepsins. Because the cysteine cathepsin family shares considerable homology and broad substrate specificities (69), functional redundancy may obscure the contribution of any one cathepsin. Therefore, the lack of a phenotype in any single cathepsin knockout does not rule out the involvement of that cathepsin or other cathepsins.

Second, as we show in this study, the inhibitor Ca074Me actually inhibits multiple cathepsins in living cells at the concentrations used in prior studies of NLRP3 activation (2, 7, 17, 20, 21, 25–29, 41–46). In fact, we found that, at doses where Ca074Me is cathepsin B specific, it does not block NLRP3-dependent IL-1β secretion; at higher doses where it inhibits multiple cathepsins, its blockade of IL-1β secretion is maximal. Indeed, Ca074Me suppresses IL-1β secretion in cathepsin B–deficient macrophages, and we found similar results with the other cathepsin knockouts as well. Concomitant testing with K777, an orally bioavailable broad spectrum inhibitor of cathepsins (65, 70–75), yielded comparable results to Ca074Me. Given this new evidence, it is now clear that the broad specificity of cathepsin inhibitors (Ca074Me and K777) is concordant with a role for multiple cathepsins in particle-induced IL-1β secretion. Moreover, even if it plays an important role in NLRP3 activation under some conditions, our data indicate that cathepsin B is not essential for this response.

Importantly, we document these two confounding variables above using a recently developed activity-based probe, BMV109 (62). Although a separate report has shown that Ca074Me can inhibit cathepsins B, S, and L in live cells with a similar probe (48), to our knowledge, this is the first time that the concentration-dependent inhibition of these cathepsins, or the compensatory upregulation of cathepsin activity, has been demonstrated in parallel with an examination of IL-1β secretion. Moreover, BMV109 labels cathepsin X, which allowed us to investigate the role of this cathepsin in IL-1β secretion.

It is critical to note that, of the five cathepsins tested in this study, cathepsin X was the only one that played a nonredundant role in IL-1β secretion. Cathepsin X appeared to be uniquely required for the IL-1β response to nigericin. In fact, we show that Ca074Me potently inhibits cathepsin X, and this likely accounts for its ability to strongly suppress nigericin-induced IL-1β secretion. Unlike Ca074Me, K777 inhibits cathepsins S at low concentrations, and deficiency of cathepsins S upregulates cathepsin X activity. Thus, this may explain why K777 is less effective against nigericin than Ca074Me, and how its broader specificity for cathepsins paradoxically makes it a more selective inhibitor of particle-induced responses. Therefore, pharmacological suppression of IL-1β secretion induced by particular stimuli likely depends on not only on

how many but which cathepsins are inhibited and at what concentrations.

Although Ca074Me and K777 could have noncathepsin off-target effects responsible for their suppression of particle-induced IL-1β secretion, we strongly favor the interpretation that they are achieving this effect by inhibiting multiple functionally redundant cathepsins. Although we observed a minor but insignificant reduction of particle-induced IL-1β secretion in the cathepsin $BL^{-/-}$ PMs, and a small but significant reduction in the pentuple cathepsin $BCSXL^{-/-}$ PMs, we believe that the residual cathepsin activity in these cells, as shown by BMV109 labeling, could be sufficient to mediate NLRP3 activation. In fact, a recent study demonstrated that inflammasome activation is an "all-or-none" response (76), which gives credence to earlier proposals that only a few molecules of active cathepsins may be sufficient to reach a minimum threshold for inflammasome activation (47). Whether this is true or not remains to be demonstrated. However, we did find more robust genetic evidence supporting an unexpected role for cathepsins in regulating the priming phase of IL-1β secretion.

Because we could not genetically suppress cathepsin activity to the same extent as inhibitors, which further reduced IL-1β secretion by these genetically deficient cells, we adopted an alternative strategy. Instead of examining cathepsin deficiency, we evaluated the effect of cathepsin deregulation by silencing two broadly active endogenous cathepsin inhibitors, cystatins C and B. Like the cathepsin family (69), the cystatin family is large (66), as might be expected of regulators of a large family of proteases. Moreover, individual cystatins specifically regulate multiple cysteine cathepsin proteases, including B, L, and S (66). Indeed, knockdown of cystatins C and B synergistically enhanced IL-1β secretion but did so for all stimuli tested. Further analyses revealed that the increase in IL-1β secretion we observed was directly proportional to the upregulation of pro–IL-1β transcript and synthesis. In fact, reexamination of the compound cathepsin knockouts ($BL^{-/-}$ and $BCS^{-/-}$) also showed that multiple redundant cathepsins play a partial but significant role in LPS-induced pro–IL-1β synthesis. As far as we know, these findings are among the first to implicate and clarify the role of endogenous cathepsin inhibitors, cystatins, in regulating IL-1β responses.

Although an association between cystatins and inflammation has been widely reported, the mechanism underlying this association has not been established. Given this context, our evidence that both cystatin B and especially cystatin C play a role in the IL-1β response is enlightening. In fact, lower serum levels of cystatin C, considered the "dominant" cystatin (77), are associated with numerous inflammatory conditions (66), including sterile inflammatory arterial disease (78). Furthermore, cystatin B deficiency in mice exacerbated LPS-induced sepsis and elevated IL-1β levels in the serum (79). This latter study demonstrated higher caspase-1 and/or caspase-11 activity and mitochondrial ROS, suggesting that loss of cystatin B increased inflammasome activation (79). However, the authors noted that there were no signs of LMD or elevated cathepsin activity in the cytosol, and effects on pro-IL-1β were not measured. Thus, our data demonstrating that cystatin deficiency increases pro–IL-1β and NLRP3 synthesis offer a different perspective that may help to explain these results. In this context, it is interesting that other studies have shown that cystatin B interacts with cathepsin L in the nucleus (80) and that cathepsin L can play a role in NF-κB activation (81). Moreover, cystatin B–deficient macrophages have lower IL-10 expression (82), and IL-10 transcriptionally downregulates IL-1β synthesis (83).

While unexpected, our data with cystatins shed further light on the mechanism by which small molecule cathepsin inhibitors may impact IL-1β secretion by modulating pro-IL-1β synthesis. Indeed, we directly demonstrated that exogenous cathepsin inhibitors also suppress LPS-induced pro-IL-1β synthesis, and that this effect contributes substantially to their suppression of IL-1β secretion by inflammasome-activating particulates and nonparticulates. Importantly, K777 and Ca074Me reduce pro-IL-1β synthesis in response to LPS priming alone, prior to any IL-1β being secreted, and they do not affect TNF-α secretion. Thus, it is unlikely that inhibitors are reducing the autocrine-like priming of pro-IL-1β synthesis simply by suppressing TNF-α or IL-1β secretion upon stimulation. Together, these findings reiterate the importance of examining both Signal 1 and Signal 2 when interpreting inflammasome studies. In fact, a recent paper emphasized this point by demonstrating that several ROS inhibitors thought to suppress NLRP3 activation actually affect Signal 1 (15). We also find that the timing of inhibitor treatment relative to LPS priming can confirm this phenomenon. If inhibitors are added earlier with respect to LPS priming, effects on priming become more pronounced and less NLRP3-specific. In some contexts, this may actually be a therapeutically advantageous characteristic.

Our findings are consistent with a prior study demonstrating that a cathepsin B inhibitor, Z-FA-fmk, suppresses LPS signaling (84). Finding discordant results with cathepsin B-deficient cells, the authors suggested this was a non-cathepsin off-target effect. Similarly, we cannot completely exclude the possibility that the various exogenous and endogenous cathepsin inhibitors are reducing IL-1β responses through off-target effects. However, given our results, it is likely that redundant cathepsins compensated for the loss of cathepsin B, and even more likely that Z-FA-fmk is non-specific for cathepsin B. Moreover, since we observed concordant results with two chemically distinct cathepsin inhibitors, Ca074Me and K777, as well as the endogenous cathepsin inhibitors, we favor the idea that the common effect of these inhibitors on pro-IL-1β synthesis is attributable to their common cathepsin targets.

Importantly, Ca074Me and K777 were consistently more effective against NLRP3-mediated IL-1β secretion compared with that mediated by Absent in melanoma 2 via dAdT, and the effects of cystatin deficiencies were similarly biased. Therefore, it appeared that cathepsins may indeed have a role in mediating stimulus-specific/priming-independent NLRP3 activation. Although this is one interpretation, others would predict that NLRP3-mediated IL-1β secretion is particularly sensitive to the levels of pro–IL-1β or that the levels of NLRP3 itself are significantly impacted by inhibitor treatment. Given the importance of LPS priming kinetics, deducing priming-independent effects on IL-1β secretion can be achieved via prolonged priming and/or concomitant inhibition of protein synthesis. Indeed, by inhibiting further pro–IL-1β synthesis with CHX following a prolonged period of LPS priming, we showed that subsequent treatment with K777 and Ca074Me affects Signal 2, independently of Signal 1. This is consistent with our finding that cathepsin inhibition suppressed inflammasome activation, as assayed by examining cleavage of caspase-1, indicating that cathepsins may also play a role in NLRP3 activation, as originally proposed. Importantly, our data showed that cathepsins are not necessary for particle-induced lysosome disruption, although this has been suggested previously (42, 85). Recently, cathepsins have also been implicated in inducing particle-stimulated K^+ efflux (K^+ efflux is thought to be an absolute requirement for NLRP3 activation) (37) in LPS-primed macrophages. Whether cathepsins affect K^+ efflux by influencing a K^+ channel or the integrity plasma membrane (secondary to inducing LMD-dependent cell death) (42, 48, 85) is not yet clear.

Whether cathepsins play a role in Signal 1 or Signal 2, it is likely that the proteolytic activity of cathepsins is necessary, given the efficacy of inhibitors; if true, the substrate involved remains to be elucidated. Importantly, both TLR4 and NLRP3, which sequentially mediate the priming and activation of IL-1β secretion, respectively, have large leucine-rich repeats (LRRs). It is presumed the LRRs act as autoinhibitory motifs that block activation until induction of structural changes or ligand binding. In fact, cathepsin inhibitors have been used to demonstrate that cleavage of the LRRs for TLRs 3, 7, and 9 is necessary for optimal activation (86, 87). Moreover, it has also been shown that NLRP1 activation can be directly mediated by proteolytic cleavage of its LRR (88, 89) and that expression of a transgenic NLRP3 protein lacking an LRR motif makes it constitutively active (90). Although this is still all speculation, LRR-targeted cleavage of TLR4 and NLRP3 by cathepsins remains an intriguing possibility that might explain our findings.

Taken together, this study identifies a previously unappreciated role for cathepsins and cystatins in the regulation of pro–IL-1β synthesis (as well as IL-1β secretion) and provides compelling evidence that cathepsins play redundant and compensatory roles in these processes. Furthermore, we have reconfirmed that Ca074Me inhibits multiple cathepsins and demonstrate conclusively that cathepsin B is not the sole target of this agent that mediates its effect on IL-1β secretion. Moreover, we identified cathepsin X as a previously unappreciated player in nigericin-induced NLRP3 activation and raised important questions as to the relative importance of cathepsins in mediating Signal 1 and Signal 2 during particle-induced NLRP3 activation and IL-1β secretion. Finally, we have characterized a cathepsin inhibitor, K777, which selectively reduces particle-induced IL-1β responses and possesses pharmacological properties warranting its investigation as a potential anti-inflammatory therapeutic (65, 70–75). Indeed, cathepsins are tractable targets for the development of small-molecule inhibitors. Our data predict that inhibitors that broadly inhibit cathepsins, like K777, might have potential as therapeutic inhibitors of particle-induced sterile inflammation.

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Disclosures

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