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Recognition of Cytoplasmic RNA Results in Cathepsin-Dependent Inflammasome Activation and Apoptosis in Human Macrophages

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dsRNA is an important pathogen-associated molecular pattern that is primarily recognized by cytosolic pattern-recognition receptors of the innate-immune system during virus infection. This recognition results in the activation of inflammasome-associated caspase-1 and apoptosis of infected cells. In this study, we used high-throughput proteomics to identify secretome, the global pattern of secreted proteins, in human primary macrophages that had been activated through the cytoplasmic dsRNA-recognition pathway. The secretome analysis revealed cytoplasmic dsRNA-recognition pathway-induced secretion of several exosome-associated proteins, as well as basal and dsRNA-activated secretion of lysosomal protease cathepsins and cysteine protease inhibitors (cystatins). Inflammasome activation was almost completely abolished by cathepsin inhibitors in response to dsRNA stimulation, as well as encephalomyocarditis virus and vesicular stomatitis virus infections. Interestingly, Western blot analysis showed that the mature form of cathepsin D, but not cathepsin B, was secreted simultaneously with IL-18 and inflammasome components ASC and caspase-1 in cytoplasmic dsRNA-stimulated cells. Furthermore, small interfering RNA-mediated silencing experiments confirmed that cathepsin D has a role in inflammasome activation. Caspase-1 activation was followed by proteolytic processing of caspase-3, indicating that inflammasome activation precedes apoptosis in macrophages that had recognized cytoplasmic RNA. Like inflammasome activation, apoptosis triggered by dsRNA stimulation and virus infection was effectively blocked by cathepsin inhibition. In conclusion, our results emphasize the importance of cathepsins in the innate immune response to virus infection. The Journal of Immunology, 2011, 186: 3085–3092.
recently shown that thioredoxin-interacting protein links oxidative stress to inflammasome activation (29, 30).

Cathepsins are lysosomal proteases that have many immunological activities. Cathepsin B and D are functionally closely related and are the most abundant in this protein family (31–33). Maturation of these proteases proceeds through several steps. Mature cathepsin B is found as a single-chain form (29 kDa) and as a two-chain form (25 and 4 kDa) of active enzyme (34). Active cathepsin D is produced through two successive cleavages of preproenzyme, resulting in formation of a two-chain mature enzyme (14-kDa L chain and 34-kDa H chain) in lysosomes (33). Recently, cystoplasmic leakage of cathepsin B was associated with inflammasome activation (29).

We studied the role of cathepsins in the innate-immune response to cytoplasmic dsRNA in human macrophages. We provide evidence that cathepsins have a central role in the activation of inflammasome and apoptosis in response to the cytoplasmic-recognition pathway.

Materials and Methods

Human macrophages, cell stimulations, and viruses

Human macrophages were differentiated from isolated blood monocytes of healthy blood donors obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland), as described previously (14). Macrophages were transfected with a mimetic of dsRNA, polyinosinic-polycytidylic acid (poly-IC), at 10 μg/ml (Sigma-Aldrich, St. Louis, MO) using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Paisley, U.K.), according to the manufacturer’s instructions. Vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) were used at a multiplicity of infection (MOI) of 1, and were described previously (35). Cathepsin B inhibitor Ca-074 Me ([L-3-trans-(propylcarbamo- moyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester), cathepsin inhibitor III (CatIII; Z-Phe-Gly-NHO-Bz-pOMe), and cysteine protease inhibitor Est (25,35)-(trans-epoxyoxycucinyl-L-lyuamido-3-methylbutane ethyl ester) (also known as E-64d) were purchased from Calbiochem (San Diego, CA). Each sample was obtained and pooled from three separate blood donors, and the results are representatives of three independently and similarly done experiments.

Small interfering RNA experiments

After 5 d of cell culture in 12-well plates, macrophages were transfected with 100 nM nontargeting control siRNA (AllStars Negative Control siRNA, Qiagen, Hilden, Germany) and with 50 nM each of two cathepsin D siRNAs (final concentration = 100 nM) (Qiagen) by using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer’s instruction. After 4 h of siRNA treatment, fresh macrophage media were added to the cells. On the following day, the cells were left unstimulated or were transfected with poly-IC for 8 h, after which the cell-culture supernatants were collected, and total proteins were isolated for ELISA and Western blot analyses, respectively.

Quantitative real-time PCR

Total RNA was isolated using an RNaseasy Plus Mini Kit (Qiagen) and reverse transcribed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Quantitative real-time PCR was performed with an ABI PRISM 7500 Sequence Detection System applying TaqMan chemistry and Predesigned TaqMan assay primers and probes (Applied Biosystems) and PerfeCTa qPCR FastMix (Quanta Biosciences, Gaithersburg, MD). The Sequence detector system version 1.4 software was used to develop the real-time PCR data (Applied Biosystems). The relative gene-expression differences were calculated with the comparative ΔΔCT method, as described earlier (36). The results are expressed as relative units, which is a fold change in gene expression that is normalized to an endogenous reference gene (18S rRNA) and is relative to nontemplate control containing molecular-grade water instead of a cDNA sample.

Western blot analysis

Total-cell lysates were prepared as previously described (13). To analyze secreted proteins, cell-culture media were collected and concentrated with Amicon Ultra-15 centrifugal filter devices, with a cut-off of 10,000 nominal m.w. limit (Millipore, Bedford, MA). Equal volumes of the concentrated cell-culture media were separated on 15% SDS-PAGE. Western blot analysis for total-cell lysates and secreted proteins was carried out as described earlier (13). Primary Abs were purchased as follows: ASC polyclonal Ab from Millipore; caspase p20 from Sigma-Aldrich; caspase-3 p19/17 and Bid from Cell Signaling Technology (Danvers, MA); caspase-1 p10 (C-20) and cathepsin B from Calbiochem, and cathepsin D (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA). To confirm equal loading and transfer onto membranes between the samples, membranes were stripped in 0.2 M NaOH for 5 min, washed in PBS-Tween 0.05% three times, and stained with ready-to-use SYPRO Ruby Protein Blot Stain, according to the manufacturer’s instructions (Sigma-Aldrich). The major basally expressed protein band is shown as a loading control.

Secretome characterization

For secretome characterization, cell-culture supernatants were first collected and concentrated with Amicon Ultra-15 centrifugal filter devices (Millipore), as above, followed by protein purification using a 2-D Clean-Up Kit (GE Healthcare, Pittsburgh, PA). Next, proteins were separated using SDS-PAGE and visualized with silver staining. For protein identification, whole gel lanes were cut into 30 pieces of equal size, proteins were in-gel digested with trypsin, and the resulting peptides were analyzed by nanoLC-MS/MS, as described previously (35). The liquid chromatography-tandem
mass spectrometry data were searched with in-house Mascot version 2.2 through ProteinPilot 3.0 interface against the SwissProt database (version 56.12). The search criteria for Mascot searches were human-specific taxonomy, trypsin digestion with one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification, and oxidation of methionine as a variable modification. All of the reported protein identifications were statistically significant ($p < 0.05$).

**Apoptosis assay**

The percentage of apoptotic cells was assayed with APOPercentage Apoptosis Assay, according to the manufacturer’s guidelines (Biocolor Life Science Assays, County Antrim, U.K.). Photographs were taken with an Olympus DP70 Digital microscope camera, connected to an Olympus IX71 light microscope, and using software DP Controller (version 2.2.1.227) and DP Manager (2.2.1.195) (Center Valley, PA). The stained and unstained cells were manually counted, and the percentage of apoptotic cells was calculated.

**ELISA measurements**

The concentrations of secreted IL-18 were analyzed by ELISA, according to the manufacturers’ instructions (Human Medical and Biological Laboratories, Nagoya, Japan; Bender MedSystems, Vienna, Austria).

**Results**

**Inflammasome activation precedes apoptosis in human macrophages during innate-immune recognition of dsRNA**

We previously showed that cytoplasmic dsRNA recognition results in activation of inflammasome and apoptosis in human macrophages (13). These events are associated with caspase-1– and caspase-3–mediated processing of pro–IL-18 to biologically active (IL-18 p18) and inactive (IL-18 p15/16) forms of this cytokine, respectively. To analyze the kinetics of inflammasome activation, poly-IC, a mimetic of dsRNA, was transfected into cytoplasm of human macrophages, and IL-18 concentration was measured from cell-culture media with ELISA. Cytoplasmic dsRNA induced IL-18 secretion at 3 h after transfection, and the greatest levels of IL-18 were seen at 12 and 18 h after stimulation (Fig. 1A). In accordance with ELISA data, Western blot analysis of intracellular proteins showed a progressive decrease in constitutively produced pro–IL-18, as well as accumulation of biologically active forms of 18-kDa IL-18 and caspase-1 p10 beginning at 3 h after cytoplasmic dsRNA stimulation (Fig. 1B). Obvious intracellular formation of activated caspase-3, caspase-3 p19/17, and its cleavage product IL-18 p16/15 was seen at 6 h after stimulation.
with dsRNA (Fig. 1B). Two isoforms of ASC, the central component of inflammasomes, were constitutively present in human macrophages, and the expression of both isoforms was slightly enhanced in response to dsRNA transfection (Fig. 1B).

In addition to processing of proinflammatory cytokines, inflammasome activation is associated with the secretion of its central components. To characterize dsRNA-induced inflammasome activation further, we collected cell-culture supernatants, concentrated them, and performed Western blot analysis of extracellular proteins. In accordance with the presented data (Fig. 1A, 1B), cytoplasmic dsRNA elicited secretion of IL-18 p18, caspase-1 p20, and ASC 6 h after stimulation (Fig. 1C). In conclusion, our results showed that cytoplasmic dsRNA-induced inflammasome activation precedes apoptosis: caspase-1 activation and IL-18 secretion occurred earlier than did activation of caspase-3 and formation of biologically inactive IL-18 fragments.

**FIGURE 4.** Cathepsins are essential for inflammasome activation in response to cytoplasmic dsRNA stimulation. Human macrophages were left untreated or were treated with Ca-074 Me (25 μM), Est (50 or 150 μM), or CatIII (50 or 150 μM) for 0.5 h before poly-IC-t stimulation for 8 h, after which cell-culture media and total cell lysates were collected. A, C, and D, IL-18 secretion was analyzed with ELISA. B, Concentrated cell-culture media were prepared for Western blot analysis with caspase-1 p20- and ASC-specific Abs. E and F, The cells were preincubated with or without Ca-074 Me (20 μM) for 0.5 h before cytoplasmic dsRNA stimulation. Total RNA was extracted at 8 h, and the expression of IFN-β and IL-29 was analyzed with quantitative RT-PCR, as described in Materials and Methods. G and H, Human primary macrophages were subjected to control and cathepsin D specific-siRNAs (100 nM) for 24 h, after which the cells were stimulated or not with cytoplasmic dsRNA for 8 h. Subsequently, cell-culture media were collected, and total protein lysates were prepared for IL-18 ELISA measurement and Western blot analysis with cathepsin B- and cathepsin D-specific Abs, respectively.
phages, respectively (Fig. 2A, Supplemental Tables I, II). At 18 h after dsRNA transfection, we detected 424 proteins from cellulture supernatants compared with 167 proteins from control-cell supernatants (Fig. 2A, Supplemental Tables III, IV). This shows that activation of the cytoplasmic RNA-recognition pathway clearly induces protein secretion by 6 h, with more robust protein secretion at 18 h. Our data showed that dsRNA stimulation of macrophages activates conventional and unconventional protein-secretion pathways. The unconventionally secreted proteins detected at 18 h after dsRNA stimulation included cystatin A, galecin-3, IL-1R antagonist, macrophage migration inhibitory factor, and thioredoxin (38, 39). Interestingly, dsRNA stimulation of macrophages also activated secretion of several exosome-associated proteins, including actin, ADP ribosylation factor 4, clathrin, heat shock proteins, histones, integrins, Ras-related proteins, tubulin, and 14-3-3 proteins (40). Ras-related proteins secreted in response to dsRNA stimulation included Rab-1A, Rab-7a, Rab-10, Rab11B, Rab13, and Rap-1b (Fig. 2B). Furthermore, the identified proteins contained several lysosomal proteins, including cathepsin B, D, S, and Z (Fig. 2C). Cathepsin B was identified at 18 h after dsRNA stimulation, whereas cathepsin Z was identified only from the control samples. Cathepsins D and S were identified from cell-culture supernatants collected from control and dsRNA-stimulated macrophages. In addition to cathepsins, secretion of cystatins, a family of cysteine protease inhibitors (41), was seen (Fig. 2C). Secretion of cystatin A and B was detected at 6 and 18 h after dsRNA stimulation. In contrast to cystatins A and B, cystatin C was also found in the cell-culture supernatants of control cells.

**Secretion of cathepsin B and D in response to dsRNA transfection**

Secretome analysis suggested major changes in secretion of cathepsins and their regulators in macrophages activated through the cytoplasmic dsRNA-recognition pathway. To characterize their regulation in response to dsRNA stimulation in more detail, cathepsin B and D protein expression and secretion were analyzed in human macrophages by Western blotting. In the intracellular protein fraction, two mature forms of cathepsin B (29 and 25 kDa) were detected in control and cytoplasmic dsRNA-stimulated cells (Fig. 3A). There was a minor decrease in the levels of both forms of cathepsin B beginning at 3 h after cytoplasmic dsRNA stimulation. With cathepsin D, a clear decrease in 52-kDa procathepsin D was observed by 3 h after dsRNA transfection, and the intermediate-sized 46-kDa form was seen at 6 and 12 h after dsRNA stimulation. Mature 34-kDa cathepsin D was constantly present in the intracellular fraction, and dsRNA stimulation had no effect on its levels. In the extracellular-protein fraction, procathepsin D (52 kDa) was constitutively secreted in human primary macrophages, and dsRNA transfection had little effect on its secretion (Fig. 3B). Secretion of mature cathepsin B and intermediate-sized 46-kDa cathepsin D occurred at 12 h after dsRNA transfection. Interestingly, secretion of mature 34-kDa cathepsin D into extracellular medium was seen 6 h after dsRNA stimulation (Fig. 3B). Importantly, the secretion of mature 34-kDa cathepsin D was simultaneous with the secretion of inflammasome components ASC and caspase-1 (Fig. 1C).

**Cathepsins are essential for inflammasome activation**

Inflammasome activation has been connected to cytoplasmic leakage of cathepsins in response to crystalline structures and microbial infections (42, 43). Thus, we next analyzed the possible role of cathepsins in cytoplasmic dsRNA-induced inflammasome activation and antiviral cytokine response with cathepsin inhibitors Ca-074 Me, a Ca-074–derivate Est, and CatIII. Ca-074 Me dramatically prevented secretion of biologically active forms of IL-18 (Fig. 4A), ASC, and caspase-1 p20 in response to dsRNA stimulation (Fig. 4B). Similarly, Est and CatIII clearly decreased dsRNA-induced IL-18 secretion in human macrophages (Fig. 4C, 4D). To study whether the antiviral cytokine response is regulated by cathepsins, macrophages were transfected with dsRNA in the absence or presence of Ca-074 Me for 8 h, after which RNA was extracted for quantitative RT-PCR analysis. Ca-074 Me had little effect on IFN-β and IL-29 mRNA expression in response to dsRNA stimulation (Fig. 4E, 4F).

We used the siRNA technique to study the specific role of cathepsin D in inflammasome activation. The human primary macrophages were treated with control siRNA and cathepsin D-specific siRNAs for 24 h, after which the cells were left unstimulated or were stimulated with cytoplasmic dsRNA for 8 h. Silencing of cathepsin D reduced cytoplasmic dsRNA-induced IL-18 secretion (Fig. 4G). Western blot analysis confirmed that cathepsin D protein expression was strongly decreased in cathepsin D siRNA-treated macrophages, whereas cathepsin B protein expression was not affected (Fig. 4H). In conclusion, our results suggested that cathepsins are crucial for efficient inflammasome activation, but not for the expression of antiviral cytokines, in response to cytoplasmic dsRNA stimulation in human macrophages.

**Cathepsins are required for the progression of apoptosis**

In addition to inflammasome activation, cathepsins are involved in programmed cell death (apoptosis) (32, 44). We previously showed that the cytoplasmic dsRNA-recognition pathway activates caspase-3 (13). We used the APOPercentage assay to further demonstrate the activation of apoptosis in dsRNA-treated macrophages. The assay clearly demonstrated activation of apoptosis in macrophages that were transfected with dsRNA at 18 h (Fig. 5A).

**FIGURE 5.** Cathepsins are required for the progression of apoptosis in dsRNA-stimulated macrophages. Human macrophages were stimulated in the presence or absence of Ca-074 Me (25 μM) for 0.5 h and then left untreated or treated with cytoplasmic dsRNA for 18 h and stained with APOPercentage Apoptosis Assay. A, Representative photographs of control (upper panel) and dsRNA-transfected (lower panel) cells (original magnification ×100). B, The apoptotic (purple) and non-apoptotic cells were counted, and the percentage of apoptotic cells was calculated. C and D, The cells were left unexposed or were exposed to Ca-074 Me and poly-IC-t for 8 h, as above, and total protein lysates were prepared for Western blot analysis with caspase-3 p19/17 and Bid-specific Abs.
Furthermore, Ca-074 Me significantly reduced the percentage of apoptotic cells (from 45 to 13%) during cytoplasmic dsRNA stimulation (Fig. 5B). Similarly, Western blot analysis showed that Ca-074 Me clearly inhibited cytoplasmic dsRNA-induced caspase-3 activation, the hallmark of apoptosis, as well as completely inhibited dsRNA-induced formation of the truncated variant of Bid (t-Bid), which promotes apoptosis by permeating the outer mitochondrial membrane with other proapoptotic proteins, such as Bax and/or Bad (32) (Fig. 5C, 5D).

**Cathepsins are essential for inflammasome activation and apoptosis triggered by EMCV and VSV infection**

A Picornaviridae EMCV and a Rhabdoviridae VSV are detected by MDA-5 and RIG-I, respectively (7). These RLR-dependent cytoplasmic RNA-recognition pathways are connected to inflammasome activation and apoptosis (12, 13, 45). To study whether cathepsins are important for inflammasome activation and apoptosis in response to RNA virus infection, macrophages were left untreated or were treated with Ca-074 Me for 0.5 h. After this, the cells were left uninfected or were infected with EMCV or VSV at an MOI of 1 for 8 or 16 h, and IL-18 secretion and activation of apoptosis were studied, respectively. Ca-074 Me inhibited EMCV- and VSV-induced IL-18 secretion (Fig. 6A, 6B). Similarly, Ca-074 Me clearly abolished EMCV- and VSV-induced caspase-3 activation and formation of t-Bid (Fig. 6C, 6D). In conclusion, our results showed that activation of apoptosis during viral infections is dependent on cathepsins.

**Discussion**

Innate immunity against viruses includes production of antiviral and proinflammatory cytokines and induction of apoptosis. Macrophages are the central players in the innate-immune system, and their recognition of viruses relies on PRRs. PRRs detect dsRNA, which is the most important PAMP formed during viral infection. In the present work, we used a high-throughput proteomics approach to characterize secretome, the global pattern of secreted proteins, of macrophages activated through the cytoplasmic dsRNA-recognition pathway. The proteomic analysis revealed that dsRNA stimulation of macrophages activates conventional and unconventional protein-secretion pathways in human macrophages. Especially, exosome-associated proteins were enriched in cell-culture supernatants of macrophages that were transfected with dsRNA. Secretome data also revealed secretion of lysosomal cathepsin proteases and cysteine protease inhibitors (cystatins) in macrophages that were activated through the cytoplasmic RNA-recognition pathway. Finally, we provide evidence that cathepsins are essential for inflammasome activation and apoptosis in response to dsRNA stimulation and viral infection.

IL-1β and IL-18 are proinflammatory cytokines that are essential for the initiation and maintenance of inflammation (16). IL-1β and IL-18 lack N-terminal leader signal peptides; thus, they are released from macrophages via an unconventional protein-secretion pathway (46). Cysteine protease caspase-1 processes their proforms into their biologically active equivalents in a cytoplasmic molecular structure called inflammasome (18). After this processing step, the biologically active forms of IL-1β and IL-18 are secreted by poorly defined mechanisms. The molecular composition of the activated inflammasome varies, depending on the virus that infects the cell, but it contains at least caspase-1 and a member of a pyrin domain family protein ASC. Picornaviruses, such as EMCV, and long-cytoplasmic dsRNA stretches activate caspase-1 and NLRP3-inflammasome via MDA-5, whereas VSV triggers caspase-1 independently of NLRP3 via RIG-I (45). In all cases, the specific mechanisms that activate inflammasomes are
not fully understood. Our data showed that a mimetic of long-cytoplasmic dsRNA, transfected poly-IC (poly-IC-L), activates inflammasome, which was observed as the appearance of active caspase-1 and intracellular accumulation of biologically active IL-18. This all coincided with the onset of IL-18 secretion (Fig. 1). More importantly, caspase-3 was activated with delayed kinetics compared with caspase-1 (Fig. 1B), indicating that inflammasome activation preceded the initiation of apoptosis in macrophages that had been activated through the cytosolic dsRNA-recognition pathway.

Membrane transfer is a common mode of intercellular communication between immune cells. Exosome secretion is a form of membrane transfer that is activated by various environmental signals, including TLR activation and/or stress conditions (40). Our secretome analysis revealed secretion of several exosome-associated proteins, including actin, ADP ribosylation factor 4, clathrin, heat shock proteins, histones, integrins, Ras-related proteins, tubulin, and 14-3-3 proteins, in response to dsRNA stimulation. Of these Ras-related proteins, Rab-1A, Rab-7, Rab-10, Rab11B, Rab-13, and Rap-1b are especially interesting because they are involved in protein transport and secretion (47). Interestingly, secretome analysis showed that Rab-1A, Rab-13, and Rap-1b secretion was already at 6 h after dsRNA transfection, and it was associated with secretion of inflammasome components. It was recently shown that another member of the RabGTPase family (Rab59a) binds caspase-1 and is involved in caspase-1-dependent secretion of IL-1β in response to LPS stimulation (48). Clearly, further studies are needed to clarify the role of Ras-related proteins in IL-1β and IL-18 secretion during viral infections.

Lysosomes are acidic organelles in which different metabolic end products and contents of late endosomes and phagosomes are degraded. In addition to inflammasome activation, moderate lysosomal damage leads to apoptosis. It is possible that the acidic environment and the action of hydrolyses produce apoptotic signals that induce apoptosis when released into cytoplasm. Cathepsins are the best-studied class of lysosomal hydrolases. Cathepsin B and D are closely related cysteine- and aspartate-specific proteases, respectively, and they were shown to promote programmed cell death (apoptosis) (44, 49). Reactive oxygen species-mediated translocation of cathepsins from lysosomes to cytoplasm is an early event in apoptosis; it occurs before loss of mitochondrial membrane potential, release of cytochrome c, and activation of the apoptotic caspase cascade (50, 51). Our results showed that inhibition of cathepsin activity with Ca-074 Me clearly reduced the percentage of apoptotic cells in cytoplasmic dsRNA-stimulated macrophages (Fig. 5B). Furthermore, caspase-3 activation in response to dsRNA stimulation and viral infection was significantly reduced by cathepsin inhibition (Figs. 5C, 6C, 6D). This showed that apoptosis triggered by the cytoplasmic RNA-recognition pathway is cathepsin dependent. Furthermore, Ca-074 Me completely inhibited formation of t-Bid in response to dsRNA mimic and viral stimuli (Figs. 5D, 6C, 6D). t-Bid promotes mitochondrial outer membrane permeabilization and the mitochondrial apoptosis pathway, as described above (52). Our results suggested that lysosomal cathepsins function upstream of mitochondrial damage to activate apoptosis during virus infection.

Cathepsin B and D have versatile immunological functions (i.e., they are involved in MHC class II-mediated Ag presentation and TLR signaling) (53, 54). Cathepsin research has been complicated by their functional redundancy, as exemplified by cathepsin knockout studies in mice (31, 53). Under normal conditions, lysosomal proteases are scarcely found outside cells, and their activities are controlled intracellularly (i.e., by endogenous cysteine protease inhibitors, including cystatin A, B, and C) (41). Our data showed that cystatins are secreted simultaneously with cathepsins in macrophages that are activated through the dsRNA-recognition pathway. Thus, cystatins may bind to cathepsin B and limit its extralysosomal functions. Interestingly, human genome does not encode aspartate-specific protease inhibitors, which could antagonize functions of cathepsin D (55); moreover, cathepsin D is able to inactivate cystatins (56, 57). Importantly, we found that cathepsin D was secreted earlier than cathepsin B in dsRNA-stimulated cells. Thus, it may be possible that the prior release of cathepsin D ensures cathepsin B-mediated inflammasome activation and apoptosis. The balance between the actions of cystatins and lysosomal leakage of cathepsins is likely important for the progression of both of these innate-immune reactions.

In our experiments, inflammasome activation in response to dsRNA stimulation, as well as EMCV and VSV infection, was completely abolished by cathepsin inhibitor Ca-074 Me. Ca-074 Me is a cell-permeable and irreversible inhibitor of intracellular cathepsins, and its primary target is cathepsin B. In addition to Ca-074 Me, a Ca-074-derivate Est and cathepsin inhibitor CatIII clearly reduced inflammasome activation. We found that secretion of the mature form of cathepsin D, but not that of cathepsin B, occurred simultaneously with inflammasome components ASC and caspase-1. Interestingly, silencing of cathepsin D with siRNAs reduced dsRNA-induced IL-18 secretion (Fig. 4G), demonstrating a role for cathepsin D in inflammasome activation. It is likely that cathepsins have redundant functions, and knockdown of a single cathepsin does not result in complete inhibition of inflammasome activation, as was also shown in our study. In conclusion, our results suggest an important role for cathepsins in the innate-immune response to virus infection, but further studies are required to show the precise role of different cathepsins in inflammasome activation and apoptosis.

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Disclosures

The authors have no financial conflicts of interest.

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