

Cutting Edge: Proteolytic Inactivation of Poly(ADP-Ribose) Polymerase 1 by the Nlrp3 and Nlrc4 Inflammasomes

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Caspase-mediated cleavage of the DNA damage sensor poly(ADP-ribose) polymerase 1 (PARP1) is a hallmark of apoptosis. However, it remains unclear whether PARP1 is processed during pyroptosis, a specialized cell-death program that occurs upon activation of caspase-1 in inflammasome complexes. In this article, we show that activation of the Nlrp3 and Nlrc4 inflammasomes induces processing of full-length PARP1 into a fragment of 89 kDa in a stimulus-dependent manner. Macrophages deficient for caspase-1 and those lacking the inflammasome adaptors Nlrp3, Nlrc4, and ASC were highly resistant to cleavage, whereas macrophages lacking the downstream inflammasome effector caspase-7 were partially protected. A modest, but statistically significant, reduction in Nlrp3 inflammasome-induced pyroptosis was observed in PARP1 knockout macrophages. Thus, protease-mediated inactivation of PARP1 is a shared feature of apoptotic, necrotic, and pyroptotic cells. *The Journal of Immunology*, 2010, 185: 3127–3130.

The DNA damage-repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) recognizes ssDNA breaks, dsDNA breaks, cross-overs, and supercoils (1). Binding to damaged DNA catalyzes the synthesis of poly(ADP) ribose, a branched polymer of repeated ADP-ribose subunits linked by glycosidic bonds. Autocatalytic poly(ADP) ribosylation of PARP1 enhances the recruitment of DNA-repair factors to salvage DNA damage in a process that consumes NAD⁺ and ATP energy stores of the cell (2, 3). To prevent energy depletion, PARP1 is proteolytically inactivated during apoptosis and necrosis (4–8), the two best-characterized programmed cell-death programs. Executioner caspases and granzymes are responsible for PARP1 processing in apoptotic cells (4–7). Similarly, PARP1 is proteolytically inactivated by lysosomal cathepsins during necrosis (8). Moreover, PARP1 cleavage fragments were demonstrated to act as dominant negative molecules preventing DNA repair by full-length PARP1; thus, they contribute to efficient cell-death execution (7). However, it

remains unclear whether PARP1 is processed during pyroptosis, a specialized form of proinflammatory programmed cell death in macrophages and dendritic cells. Pyroptosis is induced when the inflammatory caspase-1 is activated in large cytosolic protein complexes termed inflammasomes (9, 10). The Nlrp3 inflammasome represents the best-characterized caspase-1-activating complex (9). The Nod-like receptor Nlrp3 recruits caspase-1 into this complex in response to conserved microbial components, crystalline substances, and endogenous danger signals, such as ATP and uric acid (11). In contrast, the Nod-like receptor Nlrc4 is required for caspase-1 activation in macrophages infected with *Salmonella typhimurium* (9, 12, 13). The bipartite adaptor protein ASC is essential for bridging the interaction between Nod-like receptors and caspase-1 in inflammasomes, because caspase-1 activation is abolished in ASC-deficient macrophages (13, 14).

Because pyroptosis is accompanied by DNA damage and oligonucleosomal DNA fragmentation (13–17), we investigated whether PARP1 is processed during this proinflammatory cell-death mode. We found that caspase-1 and the downstream inflammasome effector caspase-7 are responsible for PARP1 cleavage during pyroptosis. PARP1-deficient macrophages were less sensitive to pyroptosis induced by activation of the Nlrp3 inflammasome, suggesting that inflammasome-mediated inactivation of PARP1 contributes to pyroptotic cell death.

Materials and Methods

Mice and macrophages

Nlrp3^{-/-}, *Nlrc4*^{-/-}, *Pycard*^{-/-}, *Casp7*^{-/-}, and *Casp1*^{-/-} mice on a C57BL/6 background were described (14, 18–20). *PARP1*^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free facility, and the animal studies were conducted under protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals. Bone marrow-derived macrophages (BMDMs) were prepared as described previously (21). Briefly, bone marrow was isolated from femurs of 6–12-wk-old mice and cultured in IMDM containing 10% heat-inactivated FBS, 20% L cell-conditioned medium, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. After 5–7 d of incubation, cells were collected and plated in 6- or 24-well plates in IMDM containing 10% heat-inactivated FBS, 100 mg/ml thymidine, and antibiotics. Macrophages were cultured for an additional 24 h before use.

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Received for publication May 12, 2010. Accepted for publication July 15, 2010.

This work was supported by National Institutes of Health Grants AR056296 and AI088177, Cancer Center Support Grant CCSG 2 P30 CA 21765, and the American Lebanese Syrian Associated Charities (to T.-D.K.). M.L. is supported by the Fund for Scientific Research-Flanders.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: BMDM, bone marrow-derived macrophage; Pam3, Pam3-GSK4; PARP1, poly(ADP-ribose) polymerase 1; WT, wild-type.

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Bacteria and microbial ligands

Salmonella enterica serovar Typhimurium cultures were grown to stationary phase under aerobic conditions at 37°C in 5 ml Luria-Bertani broth (Difco Laboratories, Franklin Lakes, NJ) and subcultured to OD₆₀₀ 0.5 before being used for infecting macrophage cultures (multiplicity of infection 5). Bacterial LPS and the TLR2 agonist Pam3-CSK4 were purchased from InvivoGen (San Diego, CA). The fungal cell wall component mannan was purchased from Sigma-Aldrich (St. Louis, MO). The ligands were used at a concentration of 10 µg/ml. ATP was from Roche (Nutley, NJ) and used at 5 mM, whereas nigericin was obtained from Sigma-Aldrich and used at 20 µM. Stimulation of BMDMs with microbial ligands, ATP, and nigericin was performed as previously described (10, 22).

Immunoblotting

Cells were washed twice with PBS and scraped in lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 5 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40) supplemented with a protease inhibitor mixture tablet (Roche). Samples were clarified, denatured with SDS buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with primary Abs and detected with a secondary anti-rabbit Ab conjugated to HRP (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by ECL (Thermo Scientific, Waltman, MA). Rabbit anti-mouse caspase-1 was a generous gift of Dr. P. Vandenaebale (Ghent University, Ghent, Belgium). PARP1 and caspase-7 Abs were from Cell Signaling Technology (Beverly, MA).

In vitro PARP-cleavage assays

rPARP1, which was purified to near homogeneity (Trevigen, Gaithersburg, MD), was subjected to in vitro protease assay in a total reaction volume of 50 µl. The reaction contents were incubated at 37°C in the presence of 30 nM caspase-1 or caspase-7 in protease assay buffer (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, and 1/100th/1 ml buffer complete protease inhibitor mixture tablet [Roche Applied Science, Penzberg, Germany]). The reactions were stopped by adding an equal volume of 2× SDS buffer, and the mixture was boiled for 5 min. The resulting cleavage products were analyzed by SDS-PAGE and by immunoblotting with anti-PARP1 Abs.

Cell-death assay

Induction of pyroptosis was quantified, according to the manufacturer's instructions, by monitoring early membrane permeabilization using the commercial Live/Dead assay (Invitrogen, Carlsbad, CA). Data were analyzed with the Student *t* test; *p* < 0.05 was considered statistically significant.

Results and Discussion

PARP1 is cleaved and inactivated in cells undergoing apoptosis or necrosis (4–8). To determine whether PARP1 is also processed during pyroptosis, the Nlrp3 inflammasome was activated in BMDMs isolated from C57BL/6 mice through stimulation with the TLR4 ligand LPS, the TLR2 ligand Pam3-CSK4, or the fungal cell wall component mannan for 3 h, followed by 5-mM ATP treatment for another 30 min. These stimuli are known to induce activation of the Nlrp3 inflammasome and pyroptotic cell death of activated macrophages (10, 21, 22). In agreement, caspase-1 activation was observed in stimulated BMDMs but not in the untreated control setup (Fig. 1A). Unlike in untreated cells, significant pyroptotic cell death was observed in macrophages stimulated with LPS, Pam3-CSK4, or mannan combined with ATP (Fig. 1B). Notably, full-length PARP1 was processed into an 89-kDa fragment in the set-ups in which caspase-1 activation and pyroptosis induction were observed, but this did not occur in untreated macrophages (Fig. 1A). These results demonstrate that PARP1 is processed during pyroptosis following ATP-induced activation of the Nlrp3 inflammasome. ATP can be substituted for the cation ionophore nigericin to engage the Nlrp3 inflammasome (10, 23). As in activated macrophages stimulated with ATP (Fig. 1A), PARP1 cleavage was observed in BMDMs that were stimulated with a combination of LPS,

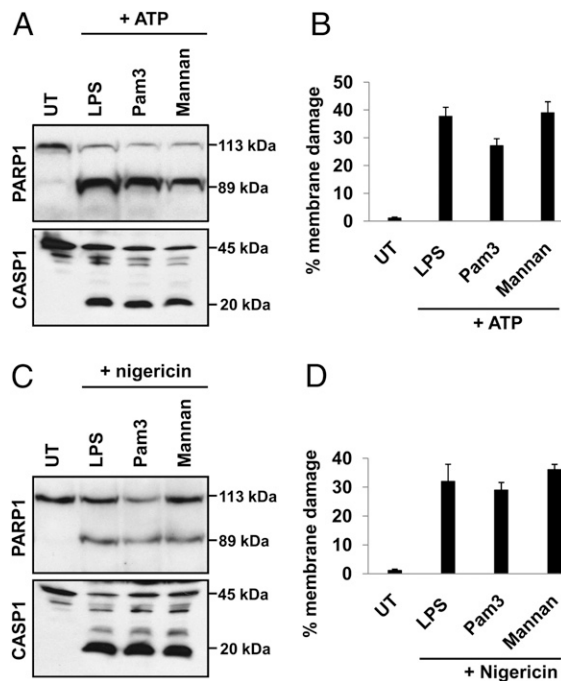


FIGURE 1. PARP1 is cleaved during ATP- and nigericin-induced pyroptotic cell death of activated macrophages. BMDMs were primed with LPS, Pam3, or mannan for 3 h and then stimulated with ATP (A, B) or nigericin (C, D) for 30 min. A and C, Cell extracts were immunoblotted with Abs against PARP1 and caspase-1. The bands corresponding to full-length PARP1 (113 kDa), the 89-kDa PARP1 fragment, procaspase-1 (45 kDa), and the large catalytic subunit (20 kDa) are indicated. B and D, Membrane permeabilization was measured as a cell-death parameter using the Live/Dead assay (Invitrogen). Cell-death data represent the mean \pm SD of triplicates. Results are representative of at least three independent experiments. Pam3, Pam3-GSK4.

Pam3-CSK4, or mannan and nigericin (Fig. 1C). PARP1 processing was accompanied by significant induction of pyroptotic cell death in nigericin-treated cells (Fig. 1D). These results demonstrate that ATP- and nigericin-mediated activation of the Nlrp3 inflammasome is associated with PARP1 cleavage.

Caspase-1 functions as the central effector of the Nlrp3 inflammasome (24) and is essential for ATP- and nigericin-induced pyroptosis in LPS-primed macrophages (10). To confirm that caspase-1 activation by the Nlrp3 inflammasome is responsible for PARP1 processing, BMDMs from mice lacking Nlrp3 (*Nlrp3*^{-/-}), the inflammasome adaptor ASC (*Pycard*^{-/-}), or caspase-1 (*Casp1*^{-/-}) were stimulated with LPS, Pam3-CSK4, or mannan in combination with ATP, as described above, and cellular lysates were probed for PARP1 processing. Although PARP1 was readily processed under these conditions in wild-type (WT) BMDMs, PARP1 cleavage was abrogated in macrophages lacking these essential components of the Nlrp3 inflammasome (Fig. 2A). In contrast to Nlrp3 and ASC, Nlr4 is not required for caspase-1 activation in TLR-activated macrophages exposed to ATP (13, 23). In agreement, PARP1 processing was not affected in Nlr4-deficient macrophages stimulated with LPS, Pam3-CSK4, or mannan in combination with ATP (Fig. 2B). Nlr4 is essential for caspase-1 activation and pyroptosis induction in macrophages infected with *Salmonella typhimurium* (12, 13). Consistently, *Salmonella*-induced PARP1 processing was abrogated in Nlr4 knockout macrophages but not in those lacking Nlrp3 (Fig. 2C). These results demonstrate that the Nlrp3 and Nlr4 inflammasomes are essential for stimulus-dependent PARP1 processing during pyroptosis.

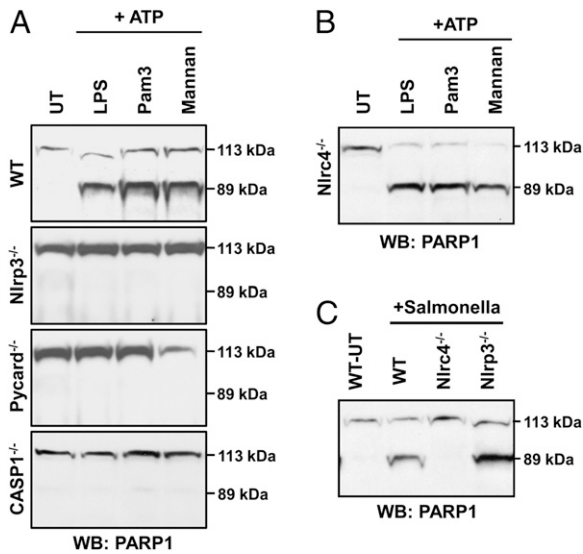


FIGURE 2. The Nlrp3 and Nlr4 inflammasomes mediate PARP1 processing during pyroptosis. Macrophages from WT, *Nlrp3*^{-/-}, *Nlr4*^{-/-}, *Pycard*^{-/-}, and *Casp1*^{-/-} mice were primed with LPS, Pam3, or mannan for 3 h and then stimulated with ATP for 30 min (A, B) or were infected for 4 h with *Salmonella* (C). Cell extracts were immunoblotted with Abs against PARP1. The bands corresponding with full-length PARP1 (113 kDa) and the 89-kDa PARP1 fragment are indicated. Results are representative of three independent experiments. Pam3, Pam3-GSK4.

It was reported that the executioner protease caspase-7 is a downstream effector of the Nlrp3 and Nlr4 inflammasomes (14, 22). In agreement, we observed caspase-7 processing, indicative of its activation, in WT macrophages that were stimulated with LPS, Pam3-CSK4, or mannan combined with ATP but not in *Casp1*^{-/-} macrophages (Fig. 3A). As expected, the caspase-7 Ab failed to detect immunoreactive bands in lysates of caspase-7-deficient (*Casp7*^{-/-}) macrophages, thus confirming its specificity (Fig. 3A). Because caspase-7 and caspase-1 are activated upon stimulation of the Nlrp3 inflammasome, both caspases may contribute to PARP1 processing during pyroptosis. To test this hypothesis, we determined the extent of PARP1 processing following Nlrp3 inflammasome activation in *Casp1*^{-/-} and *Casp7*^{-/-} macrophages. PARP1 processing was abrogated in *Casp1*^{-/-} macrophages (Fig. 3A) as a result of the defective activation of caspase-1 and caspase-7 in these cells (Fig. 3B). In contrast, PARP1 was processed in *Casp7*^{-/-} macrophages, albeit at significantly reduced levels compared with WT BMDMs (Fig. 3B). These results suggest that caspases-1 and -7 contribute to PARP1 cleavage during pyroptosis. Indeed, PARP1 was processed into an 89-kDa fragment when in vitro-translated PARP1 was incubated with recombinant caspase-1 (Fig. 3C, upper panel) or caspase-7 (Fig. 3C, lower panel). The band corresponding to full-length PARP1 gradually decreased as early as 30 min after incubation with recombinant caspase-1 or -7, whereas the 89-kDa cleavage product gained significance (Fig. 3C).

PARP1 cleavage by apoptotic caspases and granzymes is thought to contribute to efficient apoptosis execution (4–7). To determine whether PARP1 is required for efficient induction of pyroptotic cell death, BMDMs from PARP1-deficient (*PARP1*^{-/-}) mice were stimulated with LPS in combination with ATP, and the extent of pyroptosis induction was compared with WT macrophages. A modest, but statistically significant ($p < 0.004$), reduction in pyroptosis was observed in LPS-primed PARP1 knockout macrophages that were exposed to

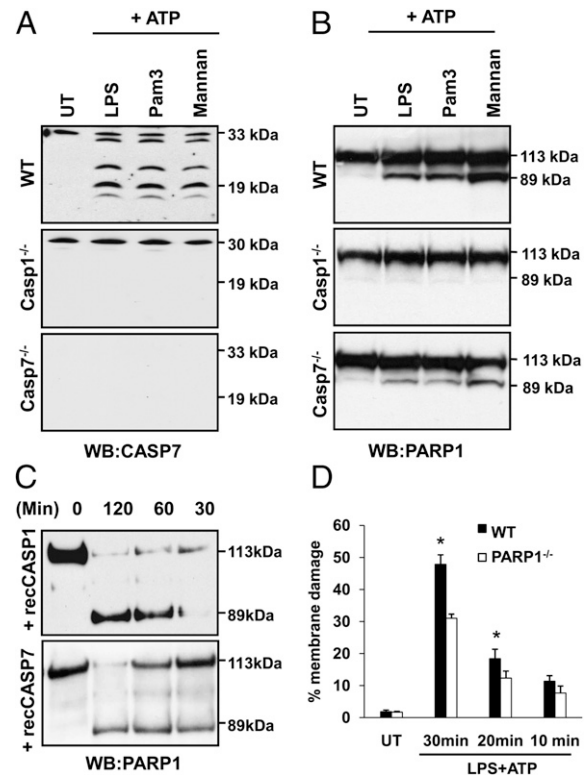


FIGURE 3. PARP1 is processed by caspases-1 and -7, and PARP1 inactivation protects against pyroptosis. A and B, Macrophages from WT *Casp1*^{-/-} and *Casp7*^{-/-} mice were primed with LPS, Pam3, or mannan for 3 h and then were stimulated with ATP for 30 min. Cell extracts were immunoblotted with Abs against caspase-7 (A) and PARP1 (B). The bands corresponding to procaspase-7 (33 kDa) and its large catalytic subunit (19 kDa), full-length PARP1 (113 kDa), and the 89-kDa PARP1 fragment are indicated. C, rPARP1 was incubated with 30 nM recombinant caspase-1 or caspase-7 in protease assay buffer (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT and complete protease inhibitor). PARP1 cleavage products were detected by Western blotting with PARP1 Ab. D, Macrophages from WT and *PARP1*^{-/-} mice were primed with LPS for 3 h and then stimulated with ATP for indicated times. Membrane permeabilization was measured as a cell-death parameter using the Live/Dead assay. Cell-death data represent the mean \pm SD of triplicates. Results are representative of three independent experiments.

5-mM ATP for 20 or 30 min (Fig. 3D). Protection from LPS+ATP-induced pyroptosis was not due to defective secretion of proinflammatory cytokines, because culture supernatants of PARP1-deficient macrophages contained normal levels of the inflammasome-dependent cytokines IL-1 β and IL-18 and the inflammasome-independent cytokines IL-6 and TNF- α (Supplemental Fig. 1). Thus, Nlrp3 inflammasome-mediated inactivation of PARP1 contributes to pyroptosis induction in activated macrophages.

Taken together, these results demonstrate that PARP1 is cleaved by caspase-1 and caspase-7 upon inflammasome activation, thus identifying one of the first molecular mechanisms by which inflammasomes induce pyroptosis. Taken together with previous reports on PARP1 processing in apoptosis (4–7) and necrosis (8), our results suggest that PARP1 processing is a general strategy used by cells undergoing programmed cell death to preserve the cellular energy stores to allow proper execution of the cell-death program and possibly to generate dominant negative cleavage fragments that may further enhance cell-death execution by inhibiting PARP1-mediated DNA repair.

Acknowledgments

We thank Anthony Coyle, Ethan Grant, John Bertin (Millennium Pharmaceuticals), Gabriel Núñez (University of Michigan), and Richard Flavell (Yale) for generously supplying mutant mice.

Disclosures

The authors have no financial conflicts of interest.

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