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*J Immunol* published online 30 January 2015

http://www.jimmunol.org/content/early/2015/01/30/jimmunol.1402951

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Supplementary Material

http://www.jimmunol.org/content/suppl/2015/01/30/jimmunol.1402951.1.DCSupplemental

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Cutting Edge: SHARPIN Is Required for Optimal NLRP3 Inflammasome Activation

Prajwal Gurung,* Mohamed Lamkanfi,†‡ and Thirumala-Devi Kanneganti*†

The NLRP3 inflammasome is a multimeric protein complex that is assembled in response to a wide array of pathogens and danger-associated molecular patterns. Despite the ability of NLRP3 to respond to diverse cues, the mechanisms controlling the assembly of this complex are contested. Recently published studies showed that HOIL-1, a member of the linear ubiquitin chain assembly complex, contributes to activation of the NLRP3 inflammasome. SHARPIN, along with HOIP and HOIL-1, constitute the linear ubiquitin chain assembly complex. In this study, we examined whether SHARPIN is required for the activation of the NLRP3 inflammasome. Using Sharpincpdm macrophages (deficient in SHARPIN expression), we demonstrate that SHARPIN is required for optimal activation of the NLRP3 inflammasome by both canonical and noncanonical stimuli. Furthermore, Sharpincpdm macrophages had dramatic effects on both the NF-κB and MAPK pathways, suggesting a role in transcriptional priming of the NLRP3 inflammasome. In conclusion, our study identified SHARPIN as a novel regulator of the NLRP3 inflammasome. The Journal of Immunology, 2015, 194: 000–000.

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Received for publication November 25, 2014. Accepted for publication January 9, 2015.

This work was supported in part by the European Research Council (Grant 281600 to M.L.), the National Institutes of Health (Grants AR056296, AI101935 to T.-D.K.), and the American Lebanese Syrian Associated Charities (to T.-D.K.). P.G. is a postdoctoral fellow supported by the Paul Barrett Endowed Fellowship from St. Jude Children’s Research Hospital.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow-derived macrophage; cpdm, chronic proliferative dermatitis; LDH, lactate dehydrogenase; LUBAC, linear ubiquitin chain assembly complex; MEF, mouse embryonic fibroblast; NLR, NOD-like receptor; poly(ADP-ribose), poly (deoxyadenylate-deoxythymidylate); WT, wild-type.

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the first time, to our knowledge, that SHARPIN regulates NF-κB and MAPK activation in response to TLR stimulation and controls NLRP3 inflammasome activation. This study highlights the complexity of regulatory mechanisms that are in place to control the NLRP3 inflammasome and identifies SHARPIN as an additional upstream regulator that potentially can be targeted for controlling aberrant NLRP3 inflammasome activation.

Materials and Methods

Mice

C57BL/6 wild-type (WT) and Sharpin<sup>ΔΔN</sup> mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at St. Jude Children’s Research Hospital. Animal studies were conducted under protocols approved by St. Jude Children’s Research Hospital and Ghent University’s Committee on the Use and Care of Animals.

Western blotting and cytokine analysis

BMDMs were prepared and stimulated, as described previously (10). Samples for immunoblotting were prepared by combining cell lysates with culture supernatants, as described previously (11). Cytokine and chemokine concentrations were determined using multiplex ELISA (Millipore). IL-1β (eBioscience) and IL-18 (MBL International) concentrations were determined by classical ELISA. Lactate dehydrogenase (LDH) release in the supernatants was determined by LDH release assay kit (Promega).

Real-time PCR

Transcript levels of Nlrc3, proIl1b, and Tnfa were quantified, as described previously (10). β-actin expression was used for normalization, and the results are presented as fold induction over levels in untreated control cells.

Statistics

GraphPad Prism 5.0 software was used for data analysis. Data are shown as mean ± SEM.

Results and Discussion

Cpdm mice were first described as C57BL/6 mice that acquired a spontaneous mutation that resulted in severe inflammation of skin and other epithelial tissues (12). In 2007, these mice were renamed “Sharpin<sup>ΔΔN</sup>” after the discovery that the phenotype was caused by a nonsense mutation in the Sharpin gene that resulted in full SHARPIN deficiency (6).

To study the role of SHARPIN in NLRP3 inflammasome activation, we generated macrophages from the bone marrow of WT and Sharpin<sup>ΔΔN</sup> mice and stimulated these macrophages with LPS (TLR4 agonist) or Pam3CSK4 (TLR2 agonist) for 3.5 h, followed by ATP for 30 min. TLR priming followed by ATP addition induces canonical NLRP3 inflammasome activation through caspase-1 and IL-1β cleavage.

**FIGURE 1.** Canonical NLRP3 inflammasome activation is abrogated in Sharpin<sup>ΔΔN</sup> BMDMs. WT and Sharpin<sup>ΔΔN</sup> BMDMs were stimulated with LPS (500 ng/ml) or Pam3CSK4 (1 μg/ml) for 4 h, with ATP added for the last 30 min. (A) Cell lysates probed for caspase-1, IL-1β, NLRP3, and β-actin. IL-1β (B) and IL-18 (C) levels in the cell culture supernatant. (D) Cell death determined by measuring LDH released in the supernatants. Data are mean ± SEM from four independent experiments. **p < 0.01, ****p < 0.0001, Student t test.

**FIGURE 2.** SHARPIN is dispensable for inflammasome activation during S. typhimurium infection and poly(dA:dT) transfection. WT and Sharpin<sup>ΔΔN</sup> BMDMs were infected with S. typhimurium for 2 h or transfected with 1 μg poly(dA:dT) for 4 h. (A) Cell lysates probed for caspase-1 and β-actin. IL-1β (B) and IL-18 (C) levels in the cell culture supernatant. (D) Cell death determined by LDH released in the supernatants from S. typhimurium- and poly(dA:dT)-stimulated samples. Data are mean ± SEM. All data are representative of four independent experiments. *p < 0.05, Student t test.
inflammasome activation (13). Surprisingly, we discovered that both LPS/ATP- and Pam3CSK4/ATP-induced caspase-1 activation and IL-1β cleavage were dramatically reduced in Sharpincpdm BMDMs (Fig. 1A). In agreement with these Western blotting observations, IL-1β and IL-18 levels in the supernatants of LPS+ATP-stimulated and Pam3CSK4+ATP-stimulated Sharpincpdm BMDMs were considerably lower than the levels secreted by WT macrophages (Fig. 1B, 1C). Moreover, pyroptotic cell death, as measured by LDH release in the supernatants, was at background levels in stimulated Sharpincpdm macrophages (Fig. 1D). To further examine whether the observed results were specific to macrophages, we stimulated WT and Sharpincpdm BMDCs with LPS and ATP. Canonical NLRP3 inflammasome activation was similarly abrogated in Sharpincpdm dendritic cells (Supplemental Fig. 1A). However, it is unknown whether SHARPIN is also involved in modulating NLRP3 inflammasome in other cells, such as neutrophils, T cells, and B cells, and will be studied in the future. Regardless, these observations collectively establish SHARPIN as a key regulator of canonical NLRP3 inflammasome activation.

The noncanonical NLRP3 inflammasome requires caspase-11 for the activation of caspase-1 and downstream IL-1β production (14). Specifically, it was shown that cytoplasmic LPS is directly recognized by caspase-11 to activate caspase-1 and promote pyroptotic cell death (15). C. rodentium infection of macrophages activates the noncanonical NLRP3 inflammasome (14). To ascertain whether SHARPIN is also required for noncanonical NLRP3 inflammasome activation, WT and Sharpincpdm BMDMs were infected with C. rodentium. Caspase-1 activation and subsequent IL-1β and IL-18 production were all reduced substantially in the absence of SHARPIN, demonstrating the importance of SHARPIN in the regulation of the noncanonical NLRP3 inflammasome (Supplemental Fig. 1B). Interestingly, caspase-11 expression was reduced slightly in Sharpincpdm BMDMs compared with WT BMDMs. Altogether, these results establish SHARPIN as a central regulator of canonical and noncanonical NLRP3 inflammasome activation.

To examine whether SHARPIN is specifically required for NLRP3 inflammasome activation, we analyzed the levels of Salmonella enterica typhimurium–induced NLRC4 and poly(deoxyadenylic-deoxythymidylic) (poly(dA:dT)-induced AIM2 inflammasome activation in WT and Sharpincpdm macrophages (16–18). S. typhimurium induced similar levels of caspase-1 activation and IL-18 production in WT and Sharpincpdm BMDMs (Fig. 2A, 2C). Similarly, poly(dA:dT) stimulated similar levels of caspase-1 activation and IL-18 production in Sharpincpdm BMDMs. These observations suggest that SHARPIN is required for the activation of the NLRP3 inflammasome but not for the activation of the NLRC4 and AIM2 inflammasomes.
WT and Sharpincpdm BMDMs (Fig. 2A, 2C). As expected, poly(dA:dT) stimulation did not induce any IL-1β production but S. typhimurium–induced IL-1β was significantly reduced in Sharpincpdm BMDMs (Fig. 2B). These results clearly demonstrate that SHARPIN is dispensable for NLRC4 and AIM2 inflammasome activation. In accordance with caspase-1 and IL-18 data, pyroptotic cell death, as measured by LDH release following S. typhimurium or poly(dA:dT) stimulation, was similar in WT and Sharpincpdm BMDMs (Fig. 2D).

Activation of the NLRP3 inflammasome requires two signals: a priming signal that upregulates expression of NLRP3 and pro–IL-1β and activation signals provided by NLRP3 agonists, such as ATP and nigericin, which trigger NLRP3 oligomerization and caspase-1 maturation. Previous studies showed that SHARPIN is required for efficient NF-κB signaling during TNF-α agonist, such as ATP Pan and nigericin, which trigger NLRP3 oligomerization and caspase-1 maturation. Previous studies showed that SHARPIN is required for efficient NF-κB signaling during TNF-α, IL-1α, CD40-, or LPS-induced signaling in MEFs and B cells, but its role in macrophages is not clear (8, 9, 14). We first evaluated whether LPS- and Pam3CSK4–induced production of IL-6, KC, and TNF-α was dependent on SHARPIN. Production of IL-6, KC, and TNF-α were considerably reduced in Sharpincpdm BMDMs, suggesting a central role for SHARPIN in TLR signaling (Supplemental Fig. 2). Remarkably, LPS-induced expression of NLRP3 and ASC was similar in WT and Sharpincpdm BMDMs (Fig. 3A). However, the expression of pro–IL-1β in Sharpincpdm BMDMs was dramatically blunted (Figs. 1A, 3A). mRNA analysis of LPS-stimulated BMDMs showed that, although Nlpr3 expression was not affected, proIl1b expression was reduced in Sharpincpdm BMDMs (Fig. 3B, 3C). Furthermore, Tysfa expression also was reduced in Sharpincpdm BMDMs (Fig. 3D). HOI-1 was recently shown to be involved in linear ubiquitination of ASC and to regulate NLRP3 inflammasome activation (3). Although a possible role for SHARPIN in linear ubiquitination of ASC cannot be excluded, our study clearly demonstrates a critical role for SHARPIN in LPS-induced priming events.

To ascertain the signaling pathways that are affected in the absence of SHARPIN, WT and Sharpincpdm BMDMs were stimulated with LPS (priming signal one required for NLRP3 inflammasome activation), and the activation of several signaling pathways was determined by Western blotting. These studies demonstrated that IkB phosphorylation and degradation were markedly reduced in Sharpincpdm BMDMs compared with WT controls (Fig. 4A). Similarly, phosphorylation of ERK1 and ERK2 MAPKs was reduced in the absence of SHARPIN (Fig. 4B). p38 MAPK phosphorylation was similarly inhibited in Sharpincpdm BMDMs (Fig. 4C). Together, these studies establish an important role for SHARPIN in regulating NF-κB, ERK, and p38 MAPK activation during LPS stimulation in BMDMs and provide a mechanistic link for regulation of the NLRP3 inflammasome.

To our knowledge, this is the first report to demonstrate a central role for SHARPIN in the activation of both the canonical and noncanonical NLRP3 inflammasome in macrophages. This regulation of the NLRP3 inflammasome by SHARPIN could be at two levels: priming and activation. Our study demonstrates that SHARPIN is required for efficient activation of NF-κB, ERK, and p38 MAPKs during the initial priming step of the NLRP3 inflammasome. Previous studies with HOI-1 (a member of the LUBAC along with HOIP and SHARPIN) showed a rather direct role for HOI-1 in linear ubiquitination of ASC and activation of the NLRP3 inflammasome. Our study shows that SHARPIN, although present in the same LUBAC complex, controls NLRP3 inflammasome activation by regulating its transcriptional priming. In conclusion, our results establish SHARPIN as a novel and central regulator of NLRP3 inflammasome signaling. Our results underscore the complexity of the NLRP3 inflammasome and uncover another molecule that could potentially be targeted to modulate and control NLRP3–associated inflammatory disorders.

Disclosures

The authors have no financial conflicts of interest.

References


SHANK-associated RH domain interacting protein (SHARPIN) is required for optimal NLRP3 inflammasome activation

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Supplementary Figure 1. SHARPIN is required for canonical and non-canonical NLRP3 inflammasome activation. A) WT and Sharpin<sup>cpdm</sup> BMDCs were stimulated with LPS (20ng/ml) or Pam3CSK4 (1µg/ml) for 4 hours with ATP for the last 30 minutes. Cell lysates were collected and immunoblotted for caspase-1, IL-1β, NLRP3 and β-actin. IL-1β and IL-18 levels in the cultured supernatants of LPS and Pam3CSK4 stimulated BMDMs. B) WT and Sharpin<sup>cpdm</sup> BMDMs were infected with <i>C. rodentium</i> for 24 hours. Gentamycin was added at 4 hours post infection to control infection. Cell lysates were harvested and immunoblotted for caspase-1, caspase-11 and β-actin. Cell supernatants were collected and IL-1β and IL-18 levels were determined. IL-6, KC and TNFα levels in the supernatants were determined in the samples supernatants. Data in A are representative of two independent experiments and data in B are cumulative of 4 independent experiments. Data are presented as mean ± SEM and student t test was used to determine statistical significance. **=p<0.01, ***=p<0.001, and ****=p<0.0001.
**Supplementary Figure 2.** LPS and Pam3CSK4 induced IL-6, KC and TNFa production is abrogated in \(\text{Sharpin}^{\text{cpdm}}\) BMDMs. WT and \(\text{Sharpin}^{\text{cpdm}}\) BMDMs were stimulated with LPS (20ng/ml) (A) or Pam3CSK4 (1mg/ml) (B) for 4 hours with ATP for the last 30 minutes. IL-6, KC and TNFa levels in the supernatants were determined using Multiplex ELISA. Data are presented as mean ± SEM and were combined from four independent experiments. Student t test was used to determine significance. *=p<0.05, **=p<0.01, and ***=p<0.001.