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Cutting Edge: SHARPIN Is Required for Optimal NLRP3 Inflammasome Activation

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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 defects 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.

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Nod-like receptors (NLRs) are cytoplasmic sensors that recognize and respond to several inflammatory triggers. Some NLRs, including NLRP1b, NLRP3, and NLRCA4, recruit bipartite adaptor protein ASC and caspase-1 to assemble multiprotein complexes known as inflammasomes (1). In particular, NLRP3 responds to a wide variety of triggers, such as ATP, the microbial toxin nigericin, uric acid crystals, as well as enteric pathogens, such as Vibrio cholerae, Escherichia coli, and Citrobacter rodentium (2). A recent study identified HOIL-1 as an integral upstream regulator of the NLRP3 inflammasome (3). In this study, the investigators suggested linear ubiquitination of ASC by HOIL-1 as the molecular mechanism for activation of the NLRP3 inflammasome (3). HOIL-1, along with HOIP and SHANK-associated RH domain interacting protein (SHARPIN), constitute the linear ubiquitin chain assembly complex (LUBAC) (4). Although it could be posited that LUBAC might be involved in the regulation of the NLRP3 inflammasome, the roles of other proteins in LUBAC have not been characterized.

The multiprotein LUBAC consists of HOIL-1, HOIP, and the recently identified component SHARPIN (4). Although HOIL-1 is required for optimal activation of NF-κB in mouse embryonic fibroblasts (MEFs), Hoil1−/− mice are phenotypically normal (5). However, mutations causing defective SHARPIN expression in chronic proliferative dermatitis (cpdm) mice (Sharpi1/pdm) result in severe hyperinflammation (6). Sharpi1/pdm mice develop chronic inflammatory dermatitis and inflammation of the gut and lungs as early as 4 wk of age (6). In addition, Sharpi1/pdm mice display underdeveloped secondary lymphoid organs, suggesting an important role for SHARPIN in the development of these organs. Similar to Hoil1−/− MEFs, Sharpi1/pdm MEFs had significantly reduced NF-κB activation in response to TNF as a result of SHARPIN’s involvement in the LUBAC (7−9). Thus, it is clear from these studies that, although both SHARPIN and HOIL-1 are components of the LUBAC, they exert different functions. Furthermore, the importance of SHARPIN in inflammasome activation has not been studied.

In this study, we used Sharpipdm macrophages to study the role of SHARPIN during inflammasome activation. We find that SHARPIN is critical for both canonical and noncanonical NLRP3 inflammasome activation but not for activation of the NLRCA4 and AIM2 inflammasomes. We further show that Sharpipdm bone marrow–derived macrophages (BMDMs) have defective activation of NF-κB, ERK1/2, and p38 MAPKs that regulate expression of components of the NLRP3 inflammasome. In conclusion, we demonstrate for
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 Sharpinpdm 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 Nlrp3, 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 “Sharpinpdm” 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 Sharpinpdm 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.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Canonical NLRP3 inflammasome activation is abrogated in Sharpinpdm BMDMs. WT and Sharpinpdm 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](http://www.jimmunol.org/)

**FIGURE 2.** SHARPIN is dispensable for inflammasome activation during S. typhimurium infection and poly(dA:dT) transfection. WT and Sharpinpdm BMDMs were infected with S. typhimurium for 2 h or transfected with 1 μg poly(dA:dT) for 4 h. (A) Cell lysates blotted for caspase-1 and β-actin. IL-1β (B) and IL-18 (C) levels in the cell culture supernatants. (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 (Fig. 2A, 2C). This indicates that SHARPIN is not required for noncanonical inflammasome activation.

![FIGURE 3.](http://www.jimmunol.org/)

**SHARPIN is required for pro-Il1b and Tnfa mRNA expression, but not for Nlrp3 mRNA expression, during LPS stimulation.** (A) WT and Sharpincpdm BMDMs were stimulated with LPS for the indicated periods of time. Cells were washed, and cell lysates were analyzed for the protein expression of NLRP3, ASC, and pro–IL-1β by Western blot. β-actin was used as a loading control. (B–D) WT and Sharpincpdm BMDMs were stimulated with LPS for 1, 2, or 4 h. RNA in the stimulated cells was harvested by the TRizol method, and the expression of Nlrp3 (B), proIl1b (C), and Tnfa (D) mRNA was determined. Data are mean ± SEM and are representative of at least three independent experiments.

![FIGURE 4.](http://www.jimmunol.org/)

**Activation of NF-κB, ERK, and p38 MAPKs is blunted in Sharpincpdm BMDMs.** (A and B) WT and Sharpincpdm BMDMs were stimulated with LPS for the indicated time. Cells were washed and lysed with RIPA buffer, and total protein concentration was quantified. Equal amount of protein samples were loaded, and the activation of IκB (A), ERK1 and ERK2 (B), and p38 MAPK (C) was determined by blotting samples for total and phosphorylated Abs. Normalized phospho-IκB was determined by taking the normalized ratio of phospho-IκB/β-actin. IκB degradation was determined by normalizing the IκB levels to 0-min IκB. Total activation of ERK1/2 and p38 was analyzed by taking normalized band intensity ratios of phosphorylated proteins/total proteins. Data are representative of at least three independent experiments.
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-1β 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–, IL-1β–, 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, Tgfa 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