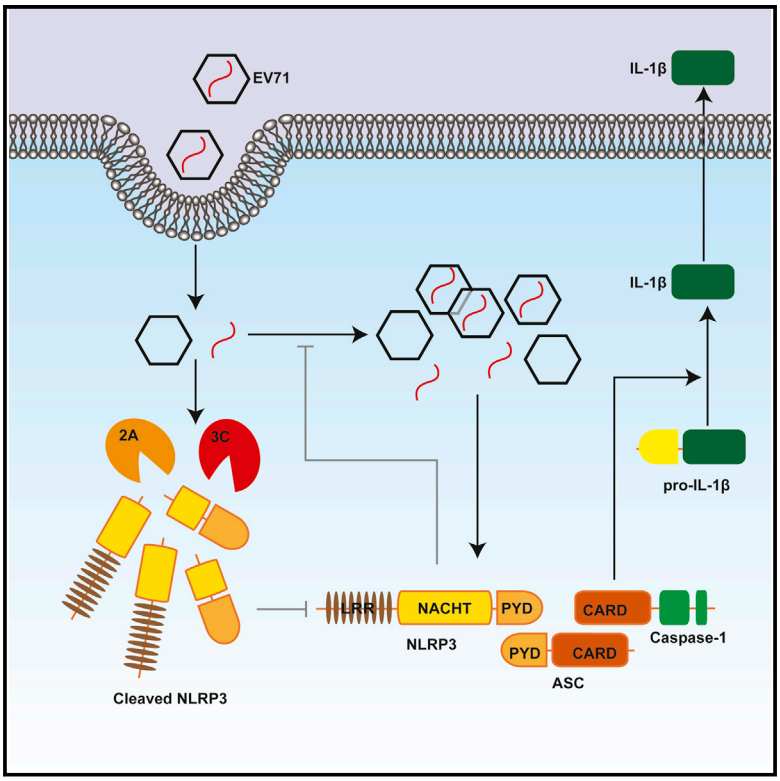
Cell Reports

Report

Reciprocal Regulation between Enterovirus 71 and the NLRP3 Inflammasome

Graphical Abstract



Highlights

- The NLRP3 inflammasome plays a protective role against EV71 infection in vivo
- EV71 replication in myeloid cells induces NLRP3 inflammasome activation
- EV71 antagonizes inflammasome activation through cleavage of NLRP3 by 2A and 3C

Authors

Hongbin Wang, Xiaobo Lei, Xia Xiao, ..., Bin He, Guangxun Meng, Jianwei Wang

Correspondence

gxmeng@sibs.ac.cn (G.M.), wangjw28@163.com (J.W.)

In Brief

Wang et al. show that the NLRP3 inflammasome plays a protective role against EV71 infection in vivo. To overcome this, EV71 has evolved strategies to counter inflammasome activation through cleavage of NLRP3.

Wang et al., 2015, Cell Reports *12*, 42–48
July 7, 2015
http://dx.doi.org/10.1016/j.celrep.2015.05.047

CellPress

OPEN ACCESS
CellPress

Cell Reports
Report

Reciprocal Regulation between Enterovirus 71 and the NLRP3 Inflammasome

Hongbin Wang,[1,3,6] Xiaobo Lei,[2,6] Xia Xiao,[2] Chunfu Yang,[1] Wenli Lu,[1] Zhong Huang,[1] Qibin Leng,[1] Qi Jin,[2] Bin He,[5] Guangxun Meng,[1,*] and Jianwei Wang[2,4,*]

[1]Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200031, China
[2]MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China
[3]University of Chinese Academy of Sciences, Beijing 100039, China
[4]Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310003, Zhejiang Province, China
[5]Department of Microbiology and Immunology, College of Medicine, University of Illinois, Chicago, IL 60612, USA
[6]Co-first author
*Correspondence: gxmeng@sibs.ac.cn (G.M.), wangjw28@163.com (J.W.)
http://dx.doi.org/10.1016/j.celrep.2015.05.047

SUMMARY

Enterovirus 71 (EV71) is the major etiological agent of hand, foot, and mouth disease (HFMD). Early studies showed that EV71-infected patients with severe complications exhibited elevated plasma levels of IL-1β, indicating that EV71 may activate inflammasomes. Our current study demonstrates that the NLRP3 inflammasome plays a protective role against EV71 infection of mice in vivo. EV71 replication in myeloid cells results in the activation of the NLRP3 inflammasome and secretion of IL-1β. Conversely, EV71 counteracts inflammasome activation through cleavage of NLRP3 by viral proteases 2A and 3C, which cleave NLRP3 protein at the G493-L494 or Q225-G226 junction, respectively. Moreover, EV71 3C interacts with NLRP3 and inhibits IL-1β secretion when expressed in mammalian cells. These results thus reveal a set of reciprocal regulations between enterovirus 71 and the NLRP3 inflammasome.

INTRODUCTION

Since its first identification in 1969, enterovirus 71 (EV71) has caused many epidemics in the world, especially in the Asia-Pacific region, manifested by hand, foot, and mouth disease (HFMD) (Huang et al., 2012). Infection with EV71 can lead to severe neurological complications such as encephalitis, acute flaccid paralysis, and pulmonary edema in children (Ooi et al., 2010). EV71 belongs to the genus *Enterovirus* within the *Picornaviridae* family, which is a non-enveloped virus with a single positive-stranded RNA genome. The genome encodes a single polyprotein precursor that gets processed into mature proteins by viral-encoded proteases 2A and 3C (Solomon et al., 2010). These proteases also cleave host factors to dampen immune responses. For example, 3C cleaves TRIF, IRF7, and IRF9 to inhibit innate immune responses (Hung et al., 2011; Lei et al., 2011, 2013b); 2A inhibits interferon responses by cleaving interferon receptor (IFNAR) and MAVS (Lu et al., 2012; Wang et al., 2013a).

Although EV71 utilizes various approaches to control immune responses, the mammalian host can still mount an effective defense against infection of this virus. Several studies showed that EV71-infected patients, particularly those with neurologic complications, carried elevated levels of cytokines and chemokines, including interleukin-1β (IL-1β), IL-6, and IL-8 (Bek and McMinn, 2010; Griffiths et al., 2012; Lin et al., 2003). Upon EV71 infection, human-monocyte-derived macrophages produce pro-inflammatory cytokines IL-1β, IL-6, and tumor necrosis factor α (TNF-α), although peripheral blood mononuclear cells (PBMCs) from patients with pulmonary edema exhibit lower IL-1β and interferon-γ (IFN-γ) levels than PBMCs from individuals with mild symptoms (Chang et al., 2006; Gong et al., 2012). These observations indicate that the inflammatory response induced by EV71 may involve the activation of inflammasomes.

The inflammasome is a multi-protein complex that senses infection or danger signals in the cell to control the maturation and secretion of the pleiotropic pro-inflammatory cytokines IL-1β and IL-18. To date, several inflammasomes have been identified, of which the NLRP3 inflammasome is the most extensively studied (Lamkanfi and Dixit, 2014). Upon activation, NLRP3 oligomerizes via homotypic interactions between NACHT domains and thereby presents clustered Pyrin domains for interaction with the Pyrin domain of ASC. Clustered ASC in turn recruits pro-caspase-1 via the CARD-CARD interaction and induces proximity-induced auto-cleavage of caspase-1 (Lu et al., 2014). Activated caspase-1 tetramer cleaves a wide range of proteins involved in various functions such as cell death and non-canonical protein secretion (Keller et al., 2008). NLRP3 inflammasome senses a plethora of pathogens and danger-associated molecular patterns (DAMPs), including environmental irritants, endogenous danger signals, and alum adjuvants (Schroder et al., 2010). Sendai virus and influenza A virus are the first reported viruses that activate the NLRP3 inflammasome (Kanneganti et al., 2006). Subsequent studies have shown that modified vaccinia virus ankara (MVA), encephalomyocarditis virus (EMCV), and adenovirus can also induce

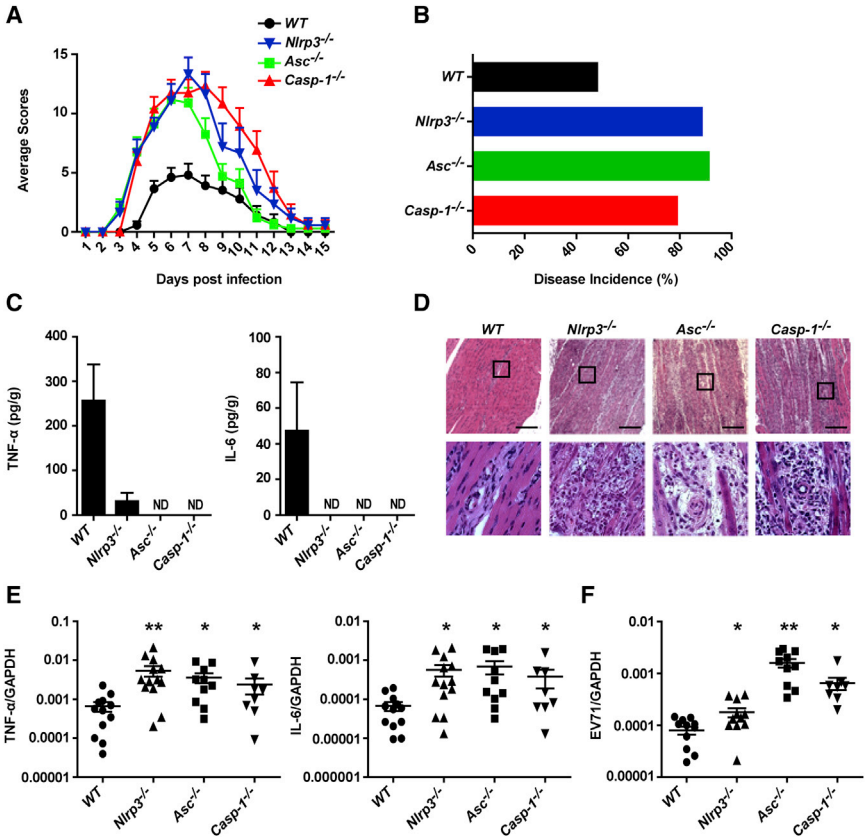


Figure 1. The NLRP3 Inflammasome Is Protective against EV71 Infection In Vivo

(A) EV71 (5×10^5 PFU) was intracerebrally (i.c.) injected into 3-day-old WT (n = 52), *Nlrp3*$^{-/-}$ (n = 26), *Asc*$^{-/-}$ (n = 34), and *Caspase-1*$^{-/-}$ (n = 52) mice. Mean scores were recorded for 15 days. Scoring method: +10, front or rear limb lameness; +20, both front and rear limb lameness; +30, death. Two-way ANOVA analysis showed that the p value was < 0.0001 when comparing the gene-deficient mice with WT control mice.

(B) Disease incidence for mice in (A).

(C) Indicated mice were i.c. infected with EV71 as in (A). 48 hr later, brain homogenates were analyzed for production of IL-6 and TNF-α via ELISA and depicted as cytokine concentrations normalized to tissue weight.

(D) Mice were infected as in (A). 9 days later, hindleg femur skeletal muscle was sectioned for H&E staining. The upper panel shows the low-scale images (scale bar, 200 μM), and the lower panel shows the high-scale image for the indicated areas from the upper panel.

(E) Real-time PCR detection of cytokine expression in the muscle from mice in (D).

(F) Real-time PCR detection of EV71 virus in the brain of mice in (D).

Values in (E) and (F) represent the mean ± SEM. Error bars represent SEM. **p < 0.01, *p < 0.05.

IL-1β secretion in an NLRP3-dependent manner (Barlan et al., 2011; Delaloye et al., 2009).

Our present study reveals that the NLRP3 inflammasome plays a protective role against EV71 infection in vivo. Furthermore, we found that EV71 proteases 2A and 3C regulate inflammasome activity through cleavage of NLRP3. Our results thus shed light on the physiopathologic relevance of the NLRP3 inflammasome during EV71 infection, which may provide therapeutic targets for anti-EV71 treatment.

RESULTS

The NLRP3 Inflammasome Protects Mice from EV71 Infection

To investigate the potential involvement of inflammasome against EV71 infection, infant mice were infected with EV71 virus through intracerebral injection to mimic neurological infection (Figure 1). In this experiment, phenotypes of C57BL/6-background mice deficient for NLRP3, ASC, or caspase-1 were compared with C57BL/6 wild-type (WT) mice. Since C57BL/6 mice are more resistant to EV71 infection than ICR mice, we observed only a low level of death (data not shown). However, it was obvious that NLRP3-, ASC-, and caspase-1-deficient mice clearly exhibited more severe disease than WT mice according to a scoring method (Figure 1A). The mice deficient for NLRP3 inflammasome showed earlier occurrence of disease, stronger paralysis, and delayed recovery compared with WT animals. The morbidity rates of these gene-deficient mice were also higher than those of WT control mice (Figure 1B).

It is known that inflammasome activation is required for secretion of mature IL-1β, which is not only a pyrogen by itself but also a potent inducer of many pro-inflammatory cytokines and chemokines (Dinarello, 2011). Consistent with this, brain tissue from NLRP3-inflammasome-deficient mice contained not only a lower level of IL-1β but also decreased amounts of IL-6, TNF-α, and IFN-γ as compared with WT control at early time point (2 days) postinfection (Figures 1C and S1A). With disease progression, the mice developed lameness in their hindlegs. H&E staining of hindleg femur muscle from mice 9 days after infection revealed that NLRP3-inflammasome-deficient animals carried more severe necrotizing myositis and stronger inflammatory cell infiltration (Figures 1D and S1B). At this stage, the expression of inflammatory cytokines and chemokines was also higher in the muscle of mice deficient for NLRP3, ASC, or caspase-1 than in WT mice (Figures 1E and S1C). Accordingly, there was higher EV71 viral load in these deficient mice in the brain 9 days after infection (Figure 1F). These data thus indicate that the NLRP3 inflammasome plays a protective role against EV71 infection in mice.

EV71 Infection Induces IL-1β Secretion in Myeloid Cells

To explore whether EV71 activates the NLRP3 inflammasome in vitro, we infected human monocytic cell line THP-1 cells with EV71 virus. A dose-dependent IL-1β secretion was detected in

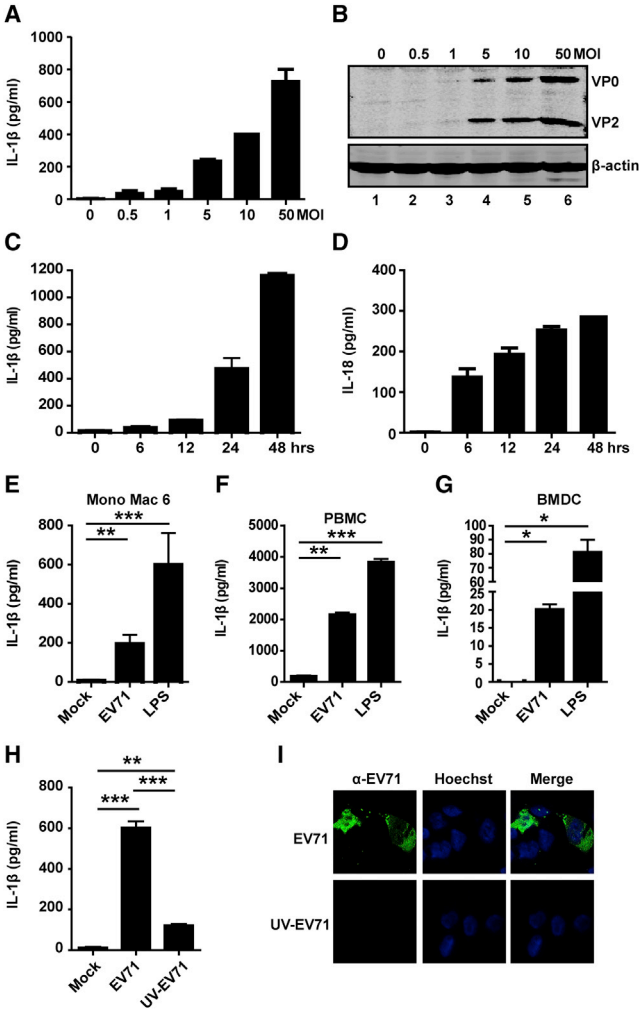


Figure 2. EV71 Infection Induces IL-1β Secretion in Myeloid Cells

(A) IL-1β secretion in THP-1 cells infected with EV71 at different multiplicities of infection (MOIs).

(B) VP0 and VP2 of EV71 expression in THP-1 cells.

(C and D) Expression of mature IL-1β and IL-18 in the supernatants of THP-1 cells treated with EV71 for different time periods.

(E–G) IL-1β secretion in Mono Mac 6 cells (E), human PBMCs (F), and mouse BMDCs (G) after treatment with EV71 or LPS.

(H) IL-1β secretion in UV-inactivated EV71-treated THP-1 cells.

(I) EV71-specific antibody staining in THP-1 cells infected with normal EV71 or UV-inactivated EV71.

Values represent the mean of triplicate samples ± SEM. Data are representative of three independent experiments. Error bars represent SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

the supernatant (Figure 2A). Accordingly, increased expression of EV71 VP0 and VP2 proteins was detected in the cell lysates, indicating an active viral replication (Figure 2B). Time-course assays also showed increased secretion of IL-1β and IL-18 (Figures 2C and 2D). Viral titer in the supernatant increased accordingly (Figure S2A). Moreover, EV71 infection of THP-1-derived macrophages, human monocytic cell line Mono Mac 6 cells, primary human PBMCs, and mouse bone-marrow-derived dendritic cells (BMDCs) also resulted in IL-1β secretion, indicating that EV71 activates the inflammasome to stimulate mature IL-1β secretion (Figures 2E–2G and S2B). These results indicate that infection with EV71 causes IL-1β secretion in myeloid cells in both humans and mice.

To establish infection, EV71 enters host cells through clathrin-dependent endocytosis (Hussain et al., 2011). We found that two clathrin-dependent endocytosis inhibitors, dynamin inhibitory peptide (DIP) and chlorpromazine (CPZ), inhibited EV71-mediated IL-1β production (Figure S2C), indicating that EV71 entry was required for IL-1β induction. Rupintrivir, an EV71 replication inhibitor, also inhibited EV71-induced IL-1β secretion (Figure S2D). Finally, UV rays or heat-inactivated EV71 virus failed to replicate or induce IL-1β secretion in THP-1 cells (Figures 2H, 2I, and S2E). Together, these results indicate that EV71 entry and replication are required for IL-1β secretion.

EV71 Infection Activates the NLRP3 Inflammasome

Since caspase-1 activation and ASC oligomerization are the direct indicators for inflammasome activation (Wang et al., 2013b), we examined the caspase-1 cleavage and ASC pyroptosome formation in EV71-infected THP-1 cells. As shown in Figures 3A and 3B, mature caspase-1 and IL-1β were detected in the supernatant and ASC oligomer was formed after EV71 infection. Lipopolysaccharide (LPS) served as a positive control in this experiment.

Moreover, we found that the inhibitor of caspase-1 AC-YVAD-CHO strongly decreased EV71-induced IL-1β secretion, indicating that EV71-induced IL-1β secretion was caspase-1 dependent (Figure 3C). In addition, high-concentration K^+ treatment blocked EV71-induced IL-1β secretion (Figure 3D). Furthermore, the NLRP3 inhibitor glibenclamide (glyburide) also inhibited EV71-induced IL-1β production (Figure 3E). To directly assess the involvement of the NLRP3 inflammasome in EV71-induced IL-1β secretion, we transfected small interfering RNAs (siRNAs) to silence the expression of ASC, NLRP3, and caspase-1 in THP-1 cells before EV71 infection (Figure S3A). EV71-induced IL-1β secretion, but not inflammasome-independent IL-8 production from knockdown cells, was clearly decreased (Figures 3F and S3B). Similar results were obtained in ASC- or NLRP3-deficient THP-1 cells (Figures 3G and S3C). Furthermore, these findings were confirmed with BMDCs from WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, or *Caspase-1*$^{-/-}$ mice. Upon EV71 infection, BMDCs deficient for NLRP3 inflammasome exhibited decreased secretion of IL-1β compared with WT cells (Figure 3H), while TNF-α production was not changed (Figure S3D). Taken together, these data indicate that EV71 infection activates the NLRP3 inflammasome and that the activity of NLRP3 is required for EV71-induced IL-1β secretion.

EV71 Proteases 2A and 3C Cleave NLRP3 and Inhibit NLRP3 Inflammasome Activation

We noticed that in NLRP3-, ASC-, and caspase-1-knockdown THP-1 cells, EV71 replication was more active, as indicated by higher viral titers in the supernatants (Figure S4A). A similar result was observed in NLRP3- or ASC-deficient THP-1 cells (Figure S4B). These data indicated that the NLRP3 inflammasome was required for the control of EV71 replication, which was consistent with our in vivo study (Figure 1). On the contrary,

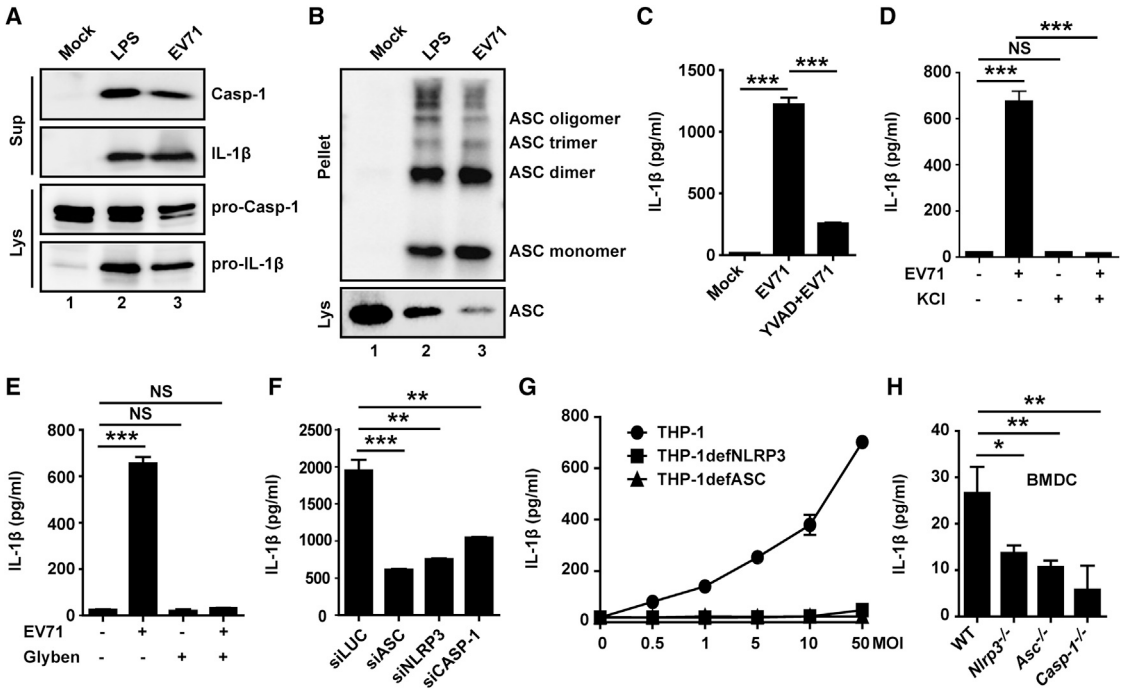


Figure 3. EV71 Activates the NLRP3 Inflammasome

(A) Detection of mature IL-1β and caspase-1 in the supernatants of THP-1 cells treated with EV71 or LPS.

(B) ASC oligomerization in THP-1-derived macrophages.

(C–E) THP-1 cells were incubated with YVAD (50 μM) (C), KCl (40 mM) (D), or glibenclamide (25 μg/ml) (E) for 1 hr and then infected with EV71 (MOI = 10) for 24 hr. Supernatants were harvested for detection of IL-1β with ELISA.

(F) IL-1β secretion in THP-1 cells expressing siRNAs targeting NLRP3, ASC, and caspase-1 after EV71 infection.

(G) THP-1, defNLRP3-THP-1, or defASC-THP-1 cells were infected with EV71 at indicated MOIs. The release of IL-1β was quantified via ELISA.

(H) BMDCs from WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Caspase-1*$^{-/-}$ mice were infected with EV71 and the supernatants were harvested for determination of IL-1β secretion via ELISA.

Values represent the mean of triplicate samples ± SEM. Data are representative of three independent experiments. Error bars represent SEM. ***p < 0.001, **p < 0.01, *p <0.05. NS, not significant.

EV71 has evolved approaches to dodge or counteract the host immune response to establish infection (Hung et al., 2011; Lei et al., 2011, 2013b; Lu et al., 2012; Wang et al., 2013a). To investigate whether EV71 3C or 2A can target NLRP3 via cleavage, recombinant 3C and 2A proteases were purified and incubated with 293T cell extracts expressing NLRP3. The 3C and 2A proteases cleaved NLRP3 protein, producing a 90-kDa or 65-kDa fragment, respectively (Figure 4A). An immunoprecipitation assay confirmed the interaction between NLRP3 and 3C when co-transfected in 293T cells (Figure S4C).

To define the cleavage site, we generated a series of human NLRP3 mutants. The mutants Q250A, Q269A, Q303A, and Q323A were still cleaved by the 3C protease (Figure 4B). However, the Q225A mutant was resistant to cleavage by 3C (lane 4, Figure 4B), suggesting the Q225-G226 pair is a cleavage site for 3C. Mouse NLRP3 also carries this cleavage site (Figure S4D) and can be cleaved by 3C (Figure S4E). Moreover, the cleavage was NLRP3 specific, since AIM2, ASC, caspase-1, and IL-1β were not cleaved by 3C (Figure S4F). Similarly, we identified the G493-L494 pair as the cleavage site for 2A (Figure 4D).

Since 3C cleaves NLRP3, it may also inhibit the activation of the NLRP3 inflammasome. To address this issue, 293T cells were transiently transfected with plasmids expressing NLRP3, ASC, pro-capase-1, and pro-IL-1β (Komune et al., 2011). It was shown that 3C expression dose-dependently reduced IL-1β secretion from 293T cells reconstituted with the NLRP3 inflammasome (Figure 4C). The cleavage of NLRP3 was correlated with 3C expression (Figure S4G). Furthermore, EV71 viral infection cleaved ectopic-expressed human and mouse NLRP3 in 293T cells (Figure 4E). Importantly, the cleavage fragments 225-N and 225-C decreased IL-1β secretion induced by LPS in THP-1 cells compared to WT-NLRP3 (Figure S4H). Moreover, expression of the cleavage-resistant Q225A mutant of NLRP3 led to clearly elevated induction of IL-1β upon EV71 infection (Figure 4F). In addition, expression of 3C in THP-1 macrophages inhibited IL-1β secretion induced by nigericin, but not poly(dA:dT) (Figure 4G), indicating that AIM2 was not targeted by 3C. Moreover, the protease activity of EV71-3C is necessary for the cleavage of NLRP3, as the 3C protease inhibitor rupintrivir, but not the caspase inhibitor Z-VAD or the proteasome inhibitor MG132, blocked 3C-mediated NLRP3 cleavage (Figure S4I). It is known that H40D or C147S substitution of 3C protein abrogates protease activity and that R84Q or V154S substitution affects its RNA binding activity. From our mutational analysis, it was found that WT 3C led to

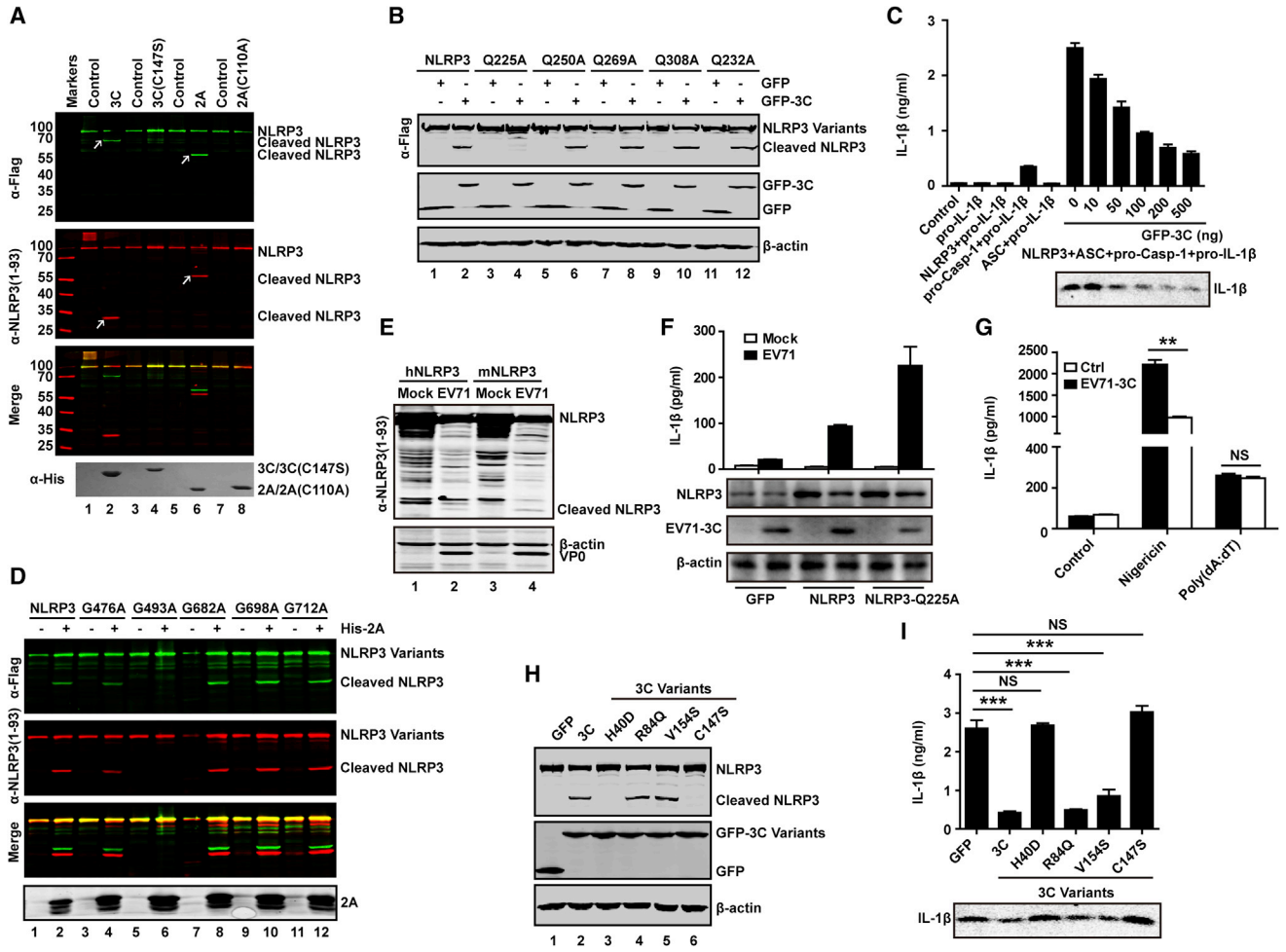


Figure 4. The Cleavage of NLRP3 by EV71 2A and 3C Proteases Inhibits NLRP3 Inflammasome Activity

(A) Cell extracts expressing NLRP3 were incubated with the recombinant 2A, 2A mutant (C110A), 3C, or 3C mutant (C147S) proteins. Samples were processed for immunoblotting using the LI-COR Odyssey dual-color system. Two antibodies that recognize NLRP3 (Flag, C-terminal of NLRP3, 800 nm, green; NLRP3, 1–93 aa, 700 nm, red) were used. An overlay of two channels is shown in the "merge" panel. Bottom panel shows the expression of 2A and 3C variants.

(B) 293T cells were transfected with wild-type NLRP3 or NLRP3 mutants as indicated along with GFP or GFP-3C. After 24 hr, cell lysates were subjected to immunoblotting.

(C) 293T cells were transfected with plasmids encoding NLRP3, pro-caspase-1, pro-IL-1β, and ASC, alone with increasing amounts of GFP-3C. 24 hr after transfection, supernatants were harvested to analyze IL-1β production via ELISA and immunoblotting.

(D) 293T cell extracts overexpressing NLRP3 variants were incubated with the recombinant 2A protease. Samples were processed for immunoblotting as in (A).

(E) 293T cells were transfected with human or mouse NLRP3. Then, cells were treated with EV71. 24 hr after infection, cell lysates were analyzed via immunoblotting.

(F) shNLRP3 THP-1 cells were transfected with GFP, NLRP3, or NLRP3-Q225A plasmids through electroporation. 48 hr later, cells were mock treated or infected with EV71 for 24 hr. IL-1β in supernatants was analyzed via ELISA. NLRP3, EV71 3C protease, and actin were detected through immunoblotting.

(G) IL-1β secretion from nigericin- or poly(dA:dT)-treated THP-1 macrophages expressing EV71-3C. Error bars represent SEM. $^{**}p < 0.01$.

(H) NLRP3 cleavage by 3C variants in 293 T cells.

(I) As in (C), except that 293T cells were transfected with GFP-3C variants alone with inflammasome plasmids. Error bars represent SEM. $^{***}p < 0.001$. NS, not significant.

NLRP3 cleavage and resulted in the inhibition of IL-1β secretion (Figures 4H and 4I). However, H40D and C147S were unable to induce NLRP3 cleavage or to inhibit IL-1β secretion. In contrast, R84Q and V154S mutation was still functioning as WT-3C (Figures 4H and 4I). Taken together, these data show that EV71 3C cleaves NLRP3 to inhibit NLRP3-mediated IL-1β secretion and that 3C protease activity plays a critical role in this process.

DISCUSSION

Although progress has been made in EV71 research since its identification, a knowledge gap exists with respect to the host immune responses against EV71 infection. Here, we demonstrate that the NLRP3 inflammasome plays a protective role against EV71 infection. At an early time point after EV71 challenge,

NLRP3-inflammasome-deficient mice showed decreased IL-1β secretion, which should be due to the lack of inflammasome activity. Moreover, the levels of other pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-6 were also lower in mice deficient for NLRP3 inflammasome compared with that in WT mice. This was probably caused indirectly by the decrease of IL-18 and IL-1β in these animals. At a later time point after EV71 infection, mice deficient for the NLRP3 inflammasome exhibited uncontrolled viral replication and elevated levels of pro-inflammatory cytokines and chemokines. This indicates that inflammasome activity is critical for the control of EV71 via initiation of an effective inflammatory response at an early time point after infection. Once EV71 establishes infection and amplifies in the host, it causes inflammation in tissues such as brain and muscle, leads to damage of these tissues in an inflammasome-independent manner, and results in pathogenesis and death. Thus, the inflammasome-dependent response early after EV71 infection seems to be beneficial to the host, while the inflammasome-independent overwhelming inflammation at a later time point is deleterious in our mouse model. Therefore, fine-tuning inflammasome activity may be helpful to control EV71 infection.

Enterovirus 71 belongs to *Picornaviruses* family, which is a large family of fast-evolving small RNA viruses including many important human and animal pathogens (Tapparel et al., 2013). As an important arm of innate immune defense, the NLRP3 inflammasome is not only critical for the control of EV71 infection, as revealed in the present study, but also involved in responses to other picornaviruses, as implied from early studies. EMCV viroporin 2B and rhinovirus ion channel protein 2B were both able to activate the NLRP3 inflammasome in cultured cells (Ito et al., 2012; Triantafilou et al., 2013). Thus, based on our current finding that the NLRP3 inflammasome is protective against EV71 infection, it is reasonable to speculate that it may also play a protective role against other picornaviruses.

Intriguingly, we find that EV71 not only activates the NLRP3 inflammasome but also inhibits inflammasome activation through cleavage of NLRP3. On the one hand, this is not surprising, because many pathogens are sensed by host innate immune system, and they also have regulatory machinery to avoid or inhibit immune responses (Taxman et al., 2010); on the other hand, this makes it hard to understand the kinetics of inflammasome activation versus inhibition upon EV71 infection. Probably, at the early phase of infection, RNA and proteins from EV71 induce NLRP3 protein expression and inflammasome assembly. When the virus replicates and generates enough 3C and 2A proteases at the later phase of infection, NLRP3 gets cleaved and inflammasome activity is harnessed. There should be overlapping time between these two phases. In fact, NLRP3 inflammasome activation by EV71 infection is much slower and weaker than that from bacterial or fungal infections (Lei et al., 2013a; Mariathasan et al., 2006). It is known that pathogens can use either competitive protein homologs or their toxic proteins to bind and inactivate inflammasome proteins (Gregory et al., 2011; LaRock and Cookson, 2012; Taxman et al., 2010). Here, we discovered a mechanism for the viral regulation of inflammasome, which is that the viral proteases cleave NLRP3 as an anti-inflammasome tactic. This may be a prevalent mechanism of various enteroviruses to combat NLRP3 inflammasome activity.

EXPERIMENTAL PROCEDURES

Mice

Asc- and *Nlrp3*-deficient mice have been described before (Mao et al., 2013; Mariathasan et al., 2004). *Caspase-1*-deficient mice were from The Jackson Laboratory and crossed onto the C57BL/6 genetic background for ten generations. Animal care and use and experimental procedures complied with national guidelines and were approved by the animal care and use committee at Institut Pasteur of Shanghai.

Statistics

Statistical evaluation between control and experimental samples was performed using the Student's t test. p values < 0.05 were considered significant (***p < 0.001, **p < 0.01, and *p < 0.05).

Detailed experimental procedures are included in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.047.

AUTHOR CONTRIBUTIONS

H.W., X.L., G.M., and J.W. designed research; H.W., X.L., X.X., C.Y., and W.L. performed the experiments; Z.H. and Q.L. contributed reagents; H.W., X.L., Q.J., B.H., G.M., and J.W. analyzed data; and H.W., X.L., G.M. and J.W. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Warren Strober for sharing *Nlrp3*-deficient mice and Dr. Vishva M. Dixit for providing *Asc*-deficient mice. This work was supported by grants from the National Key Basic Research Programs (2011CB504903, 2014CB541905, and 2015CB554302), the National Natural Science Foundation of China (81225014, 31370892, 31300712, 31270200, 31170868, and 91429307), National Major Projects for Science and Technology (2012ZX10002007-003 and 2014ZX0801011B-001), the Program for Changjiang Scholars and Innovative Research Team in Universities (IRT13007), the SA-SIBS Scholarship Program, and the CAS/SAFEA International Partnership Program for Creative Research Teams.

Received: September 10, 2014
Revised: April 13, 2015
Accepted: May 28, 2015
Published: June 25, 2015

REFERENCES

Barlan, A.U., Griffin, T.M., McGuire, K.A., and Wiethoff, C.M. (2011). Adenovirus membrane penetration activates the NLRP3 inflammasome. J. Virol. *85*, 146–155.

Bek, E.J., and McMinn, P.C. (2010). Recent advances in research on human enterovirus 71. Future Virol. *5*, 453–468.

Chang, L.Y., Hsiung, C.A., Lu, C.Y., Lin, T.Y., Huang, F.Y., Lai, Y.H., Chiang, Y.P., Chiang, B.L., Lee, C.Y., and Huang, L.M. (2006). Status of cellular rather than humoral immunity is correlated with clinical outcome of enterovirus 71. Pediatr. Res. *60*, 466–471.

Delaloye, J., Roger, T., Steiner-Tardivel, Q.G., Le Roy, D., Knaup Reymond, M., Akira, S., Petrilli, V., Gomez, C.E., Perdiguero, B., Tschopp, J., et al. (2009). Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. PLoS Pathog. *5*, e1000480.

Dinarello, C.A. (2011). A clinical perspective of IL-1β as the gatekeeper of inflammation. Eur. J. Immunol. *41*, 1203–1217.

Gong, X., Zhou, J., Zhu, W., Liu, N., Li, J., Li, L., Jin, Y., and Duan, Z. (2012). Excessive proinflammatory cytokine and chemokine responses of human monocyte-derived macrophages to enterovirus 71 infection. BMC Infect. Dis. *12*, 224.

Gregory, S.M., Davis, B.K., West, J.A., Taxman, D.J., Matsuzawa, S., Reed, J.C., Ting, J.P., and Damania, B. (2011). Discovery of a viral NLR homolog that inhibits the inflammasome. Science *331*, 330–334.

Griffiths, M.J., Ooi, M.H., Wong, S.C., Mohan, A., Podin, Y., Perera, D., Chieng, C.H., Tio, P.H., Cardosa, M.J., and Solomon, T. (2012). In enterovirus 71 encephalitis with cardio-respiratory compromise, elevated interleukin 1β, interleukin 1 receptor antagonist, and granulocyte colony-stimulating factor levels are markers of poor prognosis. J. Infect. Dis. *206*, 881–892.

Huang, H.I., Weng, K.F., and Shih, S.R. (2012). Viral and host factors that contribute to pathogenicity of enterovirus 71. Future Microbiol. *7*, 467–479.

Hung, H.C., Wang, H.C., Shih, S.R., Teng, I.F., Tseng, C.P., and Hsu, J.T. (2011). Synergistic inhibition of enterovirus 71 replication by interferon and rupintrivir. J. Infect. Dis. *203*, 1784–1790.

Hussain, K.M., Leong, K.L., Ng, M.M., and Chu, J.J. (2011). The essential role of clathrin-mediated endocytosis in the infectious entry of human enterovirus 71. J. Biol. Chem. *286*, 309–321.

Ito, M., Yanagi, Y., and Ichinohe, T. (2012). Encephalomyocarditis virus viroporin 2B activates NLRP3 inflammasome. PLoS Pathog. *8*, e1002857.

Kanneganti, T.D., Body-Malapel, M., Amer, A., Park, J.H., Whitfield, J., Franchi, L., Taraporewala, Z.F., Miller, D., Patton, J.T., Inohara, N., and Núñez, G. (2006). Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J. Biol. Chem. *281*, 36560–36568.

Keller, M., Rüegg, A., Werner, S., and Beer, H.D. (2008). Active caspase-1 is a regulator of unconventional protein secretion. Cell *132*, 818–831.

Komune, N., Ichinohe, T., Ito, M., and Yanagi, Y. (2011). Measles virus V protein inhibits NLRP3 inflammasome-mediated interleukin-1β secretion. J. Virol. *85*, 13019–13026.

Lamkanfi, M., and Dixit, V.M. (2014). Mechanisms and functions of inflammasomes. Cell *157*, 1013–1022.

LaRock, C.N., and Cookson, B.T. (2012). The Yersinia virulence effector YopM binds caspase-1 to arrest inflammasome assembly and processing. Cell Host Microbe *12*, 799–805.

Lei, X., Sun, Z., Liu, X., Jin, Q., He, B., and Wang, J. (2011). Cleavage of the adaptor protein TRIF by enterovirus 71 3C inhibits antiviral responses mediated by Toll-like receptor 3. J. Virol. *85*, 8811–8818.

Lei, G., Chen, M., Li, H., Niu, J.L., Wu, S., Mao, L., Lu, A., Wang, H., Chen, W., Xu, B., et al. (2013a). Biofilm from a clinical strain of Cryptococcus neoformans activates the NLRP3 inflammasome. Cell Res. *23*, 965–968.

Lei, X., Xiao, X., Xue, Q., Jin, Q., He, B., and Wang, J. (2013b). Cleavage of interferon regulatory factor 7 by enterovirus 71 3C suppresses cellular responses. J. Virol. *87*, 1690–1698.

Lin, T.Y., Hsia, S.H., Huang, Y.C., Wu, C.T., and Chang, L.Y. (2003). Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. Clin. Infect. Dis. *36*, 269–274.

Lu, J., Yi, L., Zhao, J., Yu, J., Chen, Y., Lin, M.C., Kung, H.F., and He, M.L. (2012). Enterovirus 71 disrupts interferon signaling by reducing the level of interferon receptor 1. J. Virol. *86*, 3767–3776.

Lu, A., Magupalli, V.G., Ruan, J., Yin, Q., Atianand, M.K., Vos, M.R., Schröder, G.F., Fitzgerald, K.A., Wu, H., and Egelman, E.H. (2014). Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. Cell *156*, 1193–1206.

Mao, K., Chen, S., Chen, M., Ma, Y., Wang, Y., Huang, B., He, Z., Zeng, Y., Hu, Y., Sun, S., et al. (2013). Nitric oxide suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock. Cell Res. *23*, 201–212.

Mariathasan, S., Newton, K., Monack, D.M., Vucic, D., French, D.M., Lee, W.P., Roose-Girma, M., Erickson, S., and Dixit, V.M. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature *430*, 213–218.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., and Dixit, V.M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. Nature *440*, 228–232.

Ooi, M.H., Wong, S.C., Lewthwaite, P., Cardosa, M.J., and Solomon, T. (2010). Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. *9*, 1097–1105.

Schroder, K., Zhou, R., and Tschopp, J. (2010). The NLRP3 inflammasome: a sensor for metabolic danger? Science *327*, 296–300.

Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M.J., McMinn, P., and Ooi, M.H. (2010). Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect. Dis. *10*, 778–790.

Tapparel, C., Siegrist, F., Petty, T.J., and Kaiser, L. (2013). Picornavirus and enterovirus diversity with associated human diseases. Infect. Genet. Evol. *14*, 282–293.

Taxman, D.J., Huang, M.T., and Ting, J.P. (2010). Inflammasome inhibition as a pathogenic stealth mechanism. Cell Host Microbe *8*, 7–11.

Triantafilou, K., Kar, S., van Kuppeveld, F.J., and Triantafilou, M. (2013). Rhinovirus-induced calcium flux triggers NLRP3 and NLRC5 activation in bronchial cells. Am. J. Respir. Cell Mol. Biol. *49*, 923–934.

Wang, B., Xi, X., Lei, X., Zhang, X., Cui, S., Wang, J., Jin, Q., and Zhao, Z. (2013a). Enterovirus 71 protease 2Apro targets MAVS to inhibit anti-viral type I interferon responses. PLoS Pathog. *9*, e1003231.

Wang, H., Mao, L., and Meng, G. (2013b). The NLRP3 inflammasome activation in human or mouse cells, sensitivity causes puzzle. Protein Cell *4*, 565–568.