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Nitric oxide suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock

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Inflammasomes are multi-protein complexes that trigger the activation of caspase-1 and the maturation of interleukin-1β (IL-1β), yet the regulation of these complexes remains poorly characterized. Here we show that nitric oxide (NO) inhibited the NLRP3-mediated ASC pyroptosome formation, caspase-1 activation and IL-1β secretion in myeloid cells from both mice and humans. Meanwhile, endogenous NO derived from iNOS (inducible form of NO synthase) also negatively regulated NLRP3 inflammasome activation. Depletion of iNOS resulted in increased accumulation of dysfunctional mitochondria in response to LPS and ATP, which was responsible for the increased IL-1β production and caspase-1 activation. iNOS deficiency or pharmacological inhibition of NO production enhanced NLRP3-dependent cytokine production *in vivo*, thus increasing mortality from LPS-induced sepsis in mice, which was prevented by NLRP3 deficiency. Our results thus identify NO as a critical negative regulator of the NLRP3 inflammasome via the stabilization of mitochondria. This study has important implications for the design of new strategies to control NLRP3-related diseases.

***Keywords*:** nitric oxide; NLRP3 inflammasome; septic shock
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Introduction

The NLRP3 inflammasome is a multi-protein complex that triggers the maturation of the pro-inflammatory cytokines IL-1β and IL-18 [1]. This complex can be activated by a broad spectrum of stimuli, including bacteria [2], viruses [3], fungi [4], components of dying cells [2] and crystal particles [5-8]. Although inflammasome activation is critical for pathogen clearance and the induction of an adaptive immune response [5, 9], dysregulated NLRP3 inflammasome activity is associated with a wide range of diseases, including cryopyrin-associated periodic syndromes [10], gout [6], type II diabetes (T2D) [11], Crohn's disease [12] and atherosclerosis [8]. As the pro-inflammatory effect of the NLRP3 inflammasome is deleterious, its activation must be tightly regulated.

Nitric oxide (NO) is a small molecule synthesized by many cell types in various tissues and is involved in multiple physiological and pathological responses, including

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circulation, blood pressure, platelet function, host defense and neurotransmission in the central nervous system and peripheral nerves [13, 14]. NO is derived from guanidino nitrogen atoms and molecular oxygen in a reaction that is catalyzed by NO synthases (NOSs) [15, 16]. There are at least three different forms of NOS expressed in various cell types: the neuronal (nNOS or NOS1) and endothelial (eNOS or NOS3) NOS that produce low levels of NO for physiological functions, whereas the inducible form of NOS (iNOS or NOS2) is activated by several immunological stimuli, such as IFN-γ, TNF-α and LPS, and generates high levels of NO [17].

NO has a variety of effects, depending on its relative concentration and the environment in which it is produced. In particular, NO has many roles in immune responses [18], including the control of infection and the regulation of signaling cascades, transcription factors, vascular responses, leukocyte rolling, migration, cytokine production and T-cell differentiation [19-23]. However, the role of NO in promoting or inhibiting septic shock remains controversial. As the NLRP3 inflammasome is critical in LPS-induced septic shock, we investigated the possible function of NO in regulating NLRP3 inflammasome activation. We found that NO negatively regulated the activation of the NLRP3 inflammasome by stabilizing mitochondria in macrophages and prevented LPS-induced septic shock *in vivo*.

Results

Nitric oxide suppresses NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation in mouse macrophages

To investigate the effect of NO on NLRP3 inflammasome activation, we stimulated lipopolysaccharide (LPS)-primed murine peritoneal macrophages with ATP, an activator of the NLRP3 inflammasome, in the presence of the NO donor SNAP or GSNO. We found that both SNAP and GSNO dramatically inhibited IL-1β secretion in a dose-dependent manner (Figure 1A and Supplementary information, Figure S1A). As L-cysteine facilitates the generation of NO from SNAP or GSNO [24], the inhibitory effects of these NO donors on IL-1β secretion were greatly enhanced in the presence of L-cysteine. In contrast, the secretion of IL-6 or TNF-α, which depends on Toll-like receptor (TLR) signaling only, was not affected by the presence of these NO donors (Figure 1A and Supplementary information, Figure S1A). Consistent with its inhibitory effect on IL-1β production, NO also dramatically suppressed caspase-1 activation (Figure 1B and Supplementary information, Figure S1B) but not the expression of the inflammasome components, NLRP3 and ASC (Figure 1B).

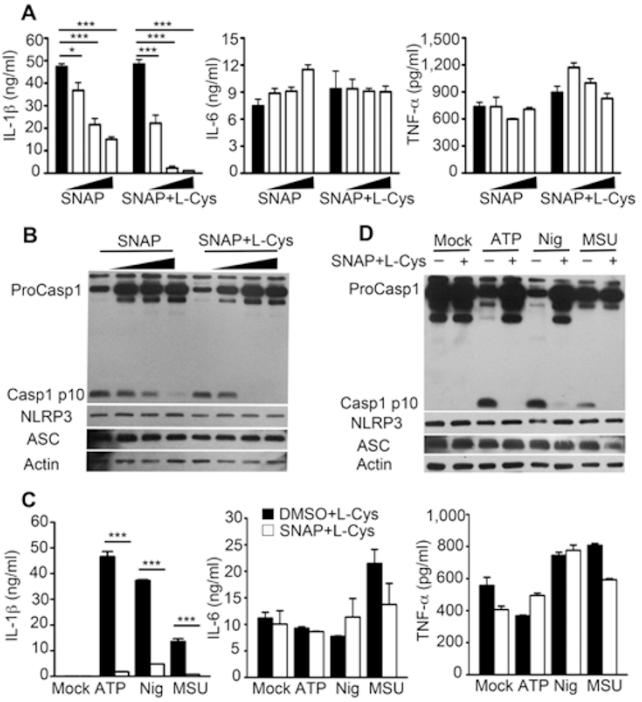

Figure 1 The NO donor SNAP inhibits NLRP3 inflammasome-mediated caspase-1 activation and IL-1β secretion. **(A)** Production of cytokines by LPS-primed peritoneal macrophages treated with different concentrations (0, 0.1, 0.5 or 1 mM) of SNAP in the presence or absence of 1 mM L-cysteine (L-Cys) for 15 min, followed by treatment with 5 mM ATP for 30 min. **(B)** Immunoblot analysis of caspase-1, NLRP3 and ASC in lysates of peritoneal macrophages treated as in **A**. **(C)** Production of cytokines by LPS-primed peritoneal macrophages treated with 0.5 mM SNAP or DMSO plus 1 mM L-Cys for 15 min and then either left unstimulated (Mock) or stimulated with 5 mM ATP (ATP, 30 min), 20 μM nigericin (Nig, 30 min) or 250 μg/ml MSU crystals (MSU, 3 h). **(D)** Immunoblot analysis of caspase-1, NLRP3 and ASC in lysates of peritoneal macrophages treated as in **C**. Actin serves as a loading control **(B** and **D)**. ProCasp1, procaspase-1; Casp1 p10, active form of caspase-1. ***P < 0.001 (Student's t-test). The data represent at least three experiments (mean ± SD in **A** and **C**).

Aside from ATP, a wide range of stimuli can activate the NLRP3 inflammasome. To examine whether NO affects NLRP3 inflammasome activation by stimuli other than ATP, we treated LPS-primed peritoneal macrophages with nigericin or monosodium urate (MSU) crystals in the presence of the NO donors SNAP or GSNO plus L-cysteine. Under these conditions, NO also inhibited IL-1β secretion and caspase-1 activation but did not affect IL-6 or TNF-α secretion (Figure 1C and 1D and Supplementary information, Figure S1C and S1D), and NLRP3 or ASC expression (Figure 1C, 1D).

As the expression of NLRP3 can be induced by other TLR agonists as well, we primed the macrophages with LPS, PGN or CpG, and then stimulated with ATP in the presence of SNAP and L-cysteine. Similar to what was observed above, NO inhibited IL-1β secretion and caspase-1 activation under different TLR stimulation conditions (Supplementary information, Figure S2A and S2B). Furthermore, we overexpressed NLRP3 in bone marrow-derived macrophages and stimulated these cells with ATP or nigericin without any priming. In this case, NO also suppressed NLRP3 inflammasome-mediated caspase-1 activation (Supplementary information, Figure S2C). These data provide clear evidence that NO suppresses NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation in mouse macrophages.

Nitric oxide inhibits human NLRP3 inflammasome activation

To investigate whether NO exerts the same anti-inflammatory effects in human cells as in mouse cells, we primed PMA-differentiated human THP-1 cells with LPS for 3 h and then stimulated the cells with nigericin or MSU crystals in the presence of SNAP and L-cysteine. As in the mouse cells, incubation with SNAP and L-cysteine effectively diminished IL-1β secretion and caspase-1 cleavage in the THP-1 cells (Figure 2A and 2B). Next, we stimulated LPS-primed human peripheral blood mononuclear cells (PBMCs) from healthy donors with nigericin or MSU crystals in the presence of SNAP and L-cysteine. Consistent with our findings in THP-1 cells, a striking decrease in IL-1β production and caspase-1 activation was noted in the primary human monocytes (Figure 2C and 2D). Thus, NO is crucial for the negative regulation of caspase-1 activation and IL-1β production in human monocytes. Collectively, these data demonstrate that the anti-inflammatory effect of NO is conserved between mice and humans.

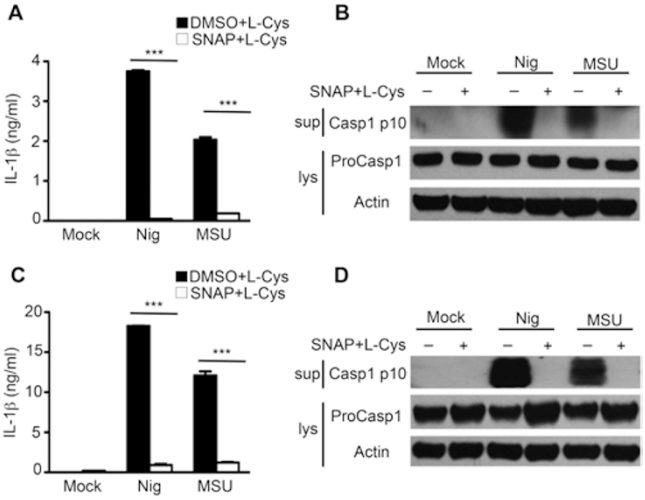

Figure 2 NO suppresses human NLRP3 inflammasome activation. **(A)** Production of IL-1β by PMA-differentiated THP-1 cells primed with 1 μg/ml LPS for 3 h, treated with 0.5 mM SNAP or DMSO plus 1 mM L-Cys for 15 min, and either left unstimulated (Mock) or stimulated with nigericin (Nig, 20 μM, 30 min) or MSU crystals (MSU, 250 mg/ml, 3 h). **(B)** Immunoblot analysis of caspase-1 in supernatants (sup), lysates (lys) of THP-1 cells treated as in **A**. **(C)** Production of IL-1β by human peripheral blood mononuclear cells (PBMCs) primed with 200 ng/ml LPS for 3 h, treated with 0.5 mM SNAP or DMSO plus 1 mM L-Cys for 15 min, and either left unstimulated (Mock) or stimulated with nigericin (Nig, 20 μM, 30 min) or MSU crystals (MSU, 250 mg/ml, 3 h). **(D)** Immunoblot analysis of caspase-1 in supernatants (sup), lysates (lys) of PBMCs treated as in **C**. Actin serves as a loading control **(B** and **D)**. ProCasp1, procaspase-1; Casp1 p10, active form of caspase-1. ***$P < 0.001$ (Student's *t*-test). The data represent at least three experiments (mean ± SD in **A** and **C**).

Endogenous NO produced by iNOS negatively regulates NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation

The findings reported above suggested that exogenous NO donors inhibited NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation. As LPS stimulation of macrophages generates large amounts of NO through the regulation of iNOS expression [17], we further evaluated the effect of endogenous NO on NLRP3 inflammasome activation. First, we found that NLRP3 inflammasome-mediated IL-1β production and caspase-1 activation varied dramatically with the duration of LPS priming. After 6 h of priming, ATP induced optimal IL-1β secretion and caspase-1 activation; this activity was clearly diminished when the LPS priming was extended to 12 h. When the LPS priming was further extended to 24 h, the ATP-induced IL-1β secretion and caspase-1 activation was nearly entirely eliminated (Figure 3A and 3B). Interestingly, the decrease in NLRP3 inflammasome activation with the prolongation of LPS priming was inversely correlated with iNOS expression, which steadily increased when the LPS stimulation was extended from 6 h to 24 h (Figure 3B). To investigate the relationship between iNOS expression and NLRP3 activation, we primed macrophages with LPS for different lengths of time in the presence or absence of the iNOS inhibitor N^G-methyl-L-arginine acetate salt (L-NMMA), followed by an ATP pulse. As shown in Figure 3A and 3B, L-NMMA dramatically enhanced IL-1β secretion and caspase-1 activation after 12 h or 24 h of LPS stimulation. Using an alternative approach, we transfected macrophages with iNOS-specific siRNA to silence the endogenous

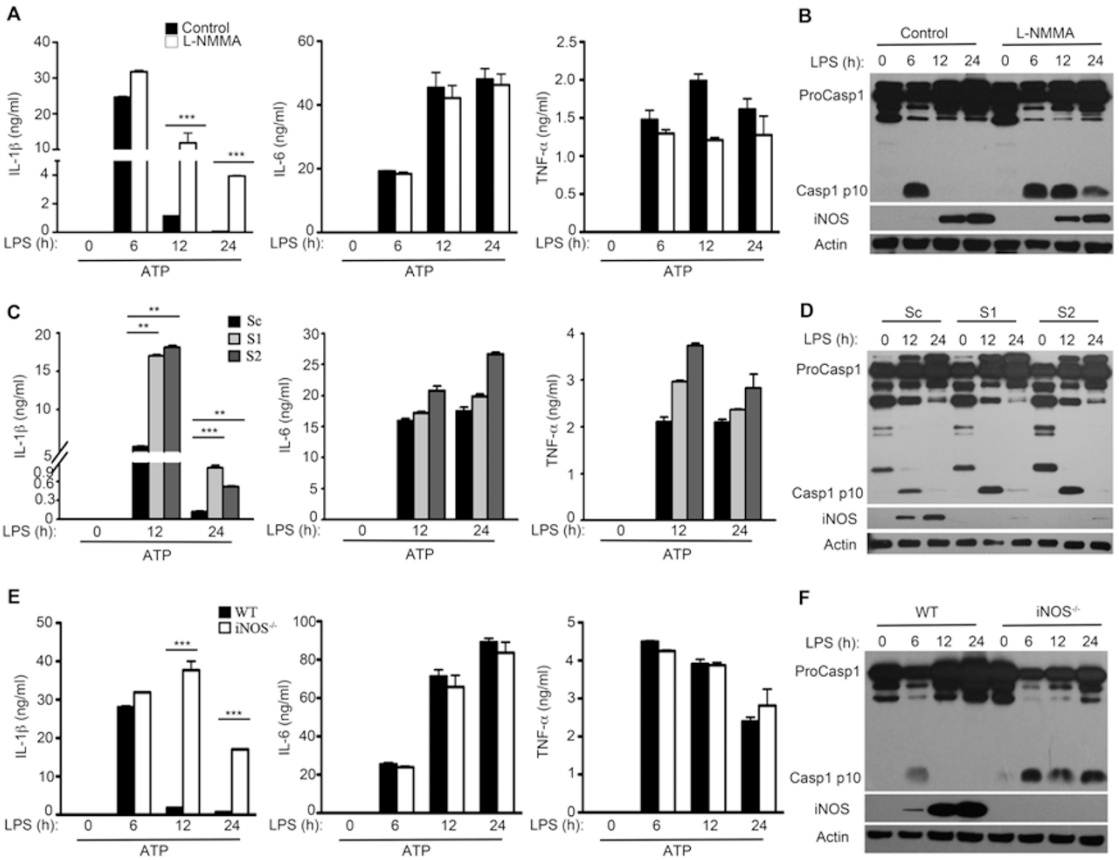

Figure 3 iNOS negatively regulates NLRP3 inflammasome activation. **(A)** Production of cytokines by peritoneal macrophages primed with LPS for different lengths of time in the presence or absence of L-NMMA, followed by stimulation with ATP for 30 min. **(B)** Immunoblot analysis of caspase-1 and iNOS in lysates of peritoneal macrophages treated as in **A**. **(C)** Production of cytokines by peritoneal macrophages transfected with iNOS-specific (S1 and S2) or control (Sc) siRNA, then primed with LPS for different lengths of time and stimulated with ATP for 30 min. **(D)** Immunoblot analysis of caspase-1 and iNOS in lysates of peritoneal macrophages treated as in **C**. **(E)** Production of cytokines by peritoneal macrophages from wild-type (WT) and iNOS-knockout ($iNOS^{-/-}$) mice primed with LPS for different lengths of time and stimulated with ATP. **(F)** Immunoblot analysis of caspase-1 and iNOS in lysates of peritoneal macrophages from WT and $iNOS^{-/-}$ mice treated as in **E**. Actin serves as a loading control **(B**, **D** and **E)**. ProCasp1, procaspase-1; Casp1 p10, active form of caspase-1. $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test). The data represent at least three experiments (mean ± SD in **A**, **C** and **E**).

expression of this enzyme. Consistent with the effect of the iNOS inhibitor L-NMMA, iNOS silencing by siRNA also significantly increased both IL-1β secretion and caspase-1 activation (Figure 3C and 3D). Finally, when the macrophages isolated from wild-type and iNOS-deficient mice were stimulated with LPS for different durations and pulsed with ATP, the iNOS deficiency completely or dramatically reversed the decrease in IL-1β secretion and caspase-1 activation that occurred in wild-type cells after 12 h or 24 h of LPS stimulation (Figure 3E and 3F). In contrast, iNOS inhibition or deficiency did not affect IL-6 or TNF-α secretion (Figure 3A, 3C and 3E). These results collectively confirm that the endogenous NO produced by iNOS negatively regulates NLRP3 inflammasome activation in macrophages.

Nitric oxide inhibits NLRP3-mediated ASC pyroptosome formation

Besides caspase-1 activation and IL-1β release, an additional marker for inflammasome activation is the formation of the ASC pyroptosome, a large structure assembled by ASC that is believed to mediate caspase-1 activation [25]. In agreement with previous reports, immunostaining for endogenous ASC in macrophages showed that over 70% of the macrophages contained the ASC pyroptosome after stimulation with LPS and ATP (71.89%), or with LPS and nigericin (76.47%). In contrast, ASC pyroptosome formation was prevented in the

presence of the NO donor SNAP plus L-cysteine (3.01% for LPS and ATP, 11.03% for LPS and nigericin) (Figure 4A and 4B).

Additionally, a biochemical assay was employed to examine NLRP3-mediated formation of the ASC pyroptosome. We isolated the ASC pyroptosome using a chemical procedure reported in a previous study [26]. As the ASC pyroptosome is ~1 μm in diameter in macrophages, centrifugation at a relatively low speed (5 000× *g*) yielded cell-free pellets of ASC pyroptosomes. This material was subjected to chemical crosslinking using the non-cleavable protein crosslinking agent disuccinimidyl suberate (DSS) to determine the extent of ASC oligomerization in the control and ATP- or nigericin-stimulated cells. As shown in Figure 4C, after ATP or nigericin stimulation, in the absence of SNAP plus L-cysteine, the ASC proteins were predominantly redistributed from the lysates to the pellets and were crosslinked by DSS to form ASC dimers, trimers and oligomers; whereas, ASC redistribution and oligomerization were completely or largely diminished in the presence of SNAP (Figure 4C). Thus, consistent with the confocal assay, the NO donor SNAP suppresses ASC redistribution and oligomerization.

It is believed that caspase-1 is activated on the ASC pyroptosome [27, 28]. Here, we detected caspase-1 distribution in macrophages stimulated with LPS plus ATP or nigericin, and found that when the NLRP3 inflammasome was activated, caspase-1 was recruited to and co-localized with the ASC pyroptosome. In contrast, NO prevented the formation of the ASC pyroptosome and the recruitment of caspase-1 (Supplementary information, Figure S3), which is consistent with the decreased caspase-1 activation observed earlier. Although NO can inhibit caspase-1 activity by direct modification [29, 30], we asked whether the inhibitory effect of NO on ASC pyroptosome formation was caspase-1 independent. Indeed, we found that caspase-1-deficient macrophages

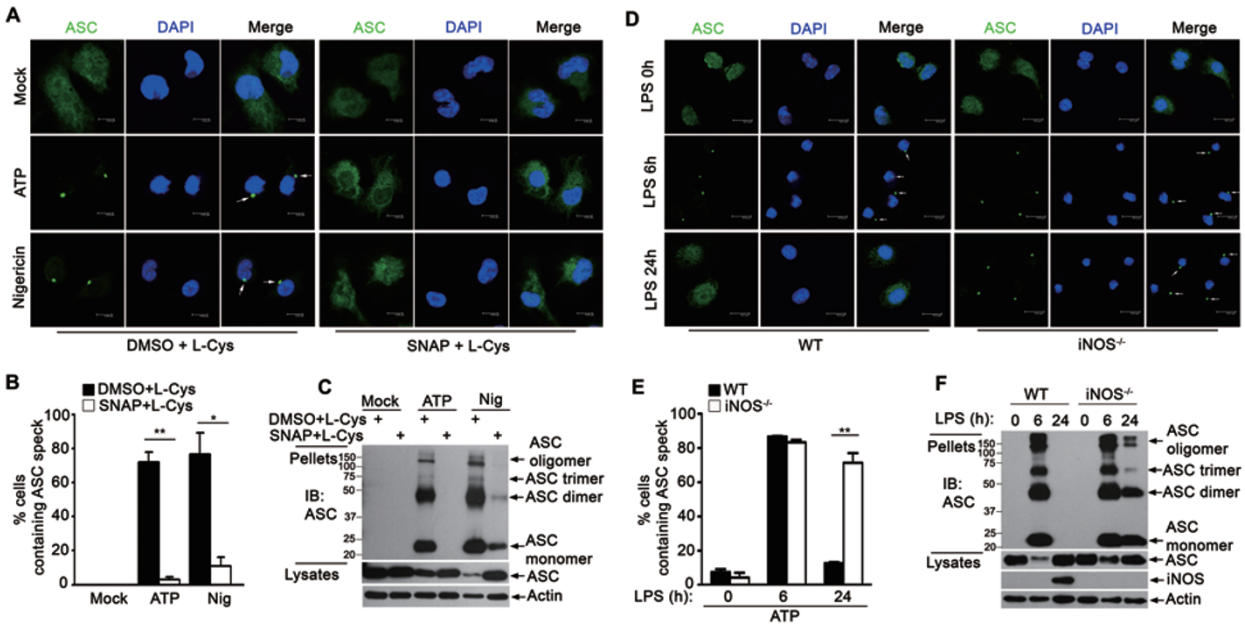

Figure 4 NO dramatically inhibits NLRP3 inflammasome-mediated ASC pyroptosome formation. **(A)** Immunofluorescence microscopy of LPS-primed peritoneal macrophages treated with 0.5 mM SNAP or DMSO plus L-Cys for 15 min, left unstimulated (Mock) or stimulated with ATP or nigericin, and then stained for ASC and DNA (with DAPI). Scale bars, 5 μm. **(B)** Percentage of macrophages containing ASC foci. The quantification represents the mean of three independent experiments, with at least 200 cells counted in each experiment. **(C)** ASC oligomerization and redistribution assay in peritoneal macrophages treated as in **A**. Immunoblot analysis of ASC in crosslinked pellets (upper panels) and in cell lysates (lower panels). **(D)** Immunofluorescence microscopy of peritoneal macrophages from WT and iNOS$^{-/-}$ mice primed with LPS for different lengths of time and stimulated with ATP, followed by staining for ASC and DNA. Scale bars, 10 μm. **(E)** Percentage of macrophages containing ASC foci. The quantification represents the mean of three independent experiments, with at least 200 cells counted in each experiment. **(F)** ASC oligomerization and redistirbution assay in peritoneal macrophages from WT and iNOS$^{-/-}$ mice treated as in **D**. Immunoblot analysis of ASC in crosslinked pellets (upper panels), ASC and iNOS in cell lysates (lower panels). Actin serves as a loading control **(C** and **F)**. $^{**}P < 0.01$ and $^{*}P < 0.05$ (Student's *t*-test). The data represent at least three experiments.

stimulated with LPS plus ATP or nigericin formed ASC pyroptosomes normally (Supplementary information, Figure S4A and S4B) and that NO still inhibited the formation of the ASC pyroptosome in these cells. These data indicate that the inhibitory effect of NO on NLRP3-mediated ASC pyroptosome formation is completely independent of caspase-1.

Next, we examined the impact of endogenous NO on NLRP3-mediated ASC pyroptosome formation. Along with IL-1β production and caspase-1 activation in wild-type macrophages, stimulation with ATP induced ASC pyroptosome formation at 6 h of LPS priming. But when the priming of LPS was extended to 24 h, the capacity to form ASC pyroptosome in wild-type macrophages receded. By contrast, in iNOS-deficient macrophages, the ASC pyroptosome was efficiently assembled at both the early (6 h) and late (24 h) stages of LPS priming when stimulated with ATP (Figure 4D, 4E and 4F). These data indicate that endogenous NO inhibits ASC pyroptosome formation in addition to NLRP3-mediated IL-1β secretion and caspase-1 activation.

iNOS deficiency promotes accumulation of damaged mitochondria

To explore the mechanism by which NO regulates NLRP3 inflammasome activation, we examined whether

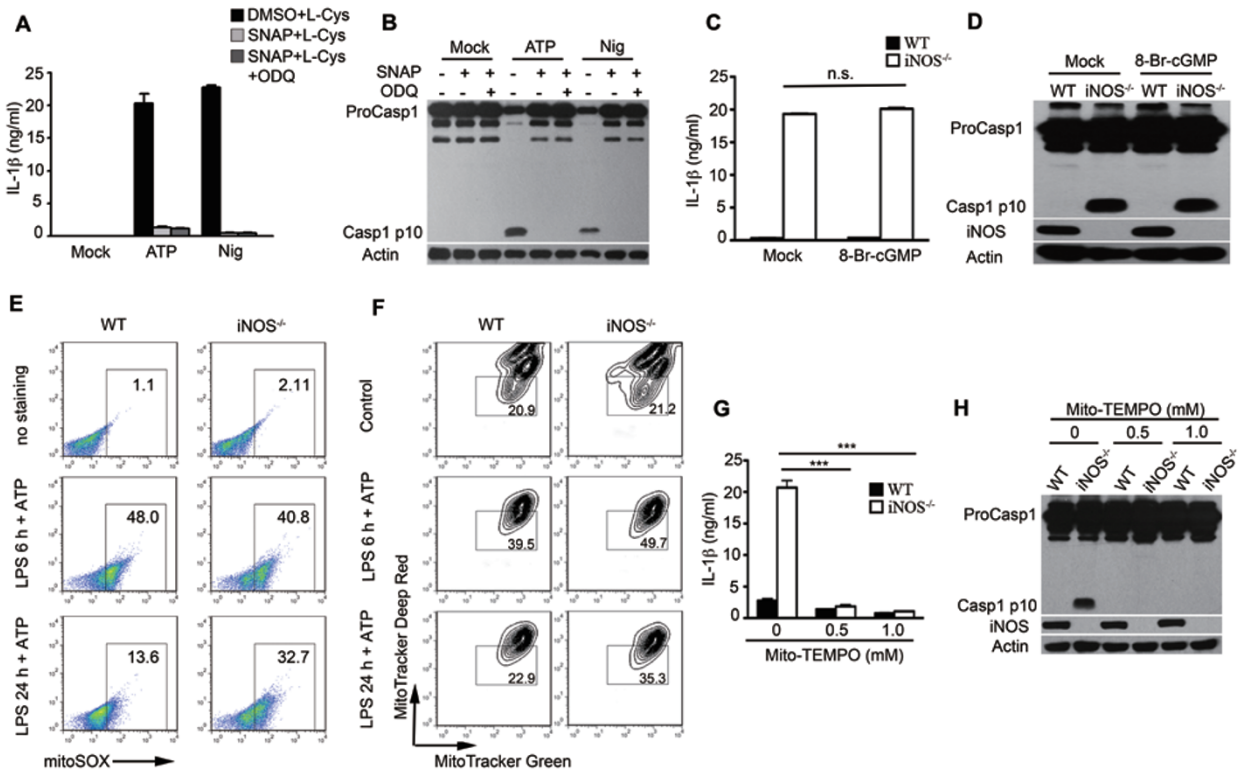

Figure 5 Increased mitochondrial ROS production and mitochondrial damage induced by LPS and ATP treatment in iNOS$^{-/-}$ macrophages are responsible for excessive NLRP3 inflammasome activation. **(A)** Production of IL-1β by LPS-primed peritoneal macrophages treated with DMSO, SNAP alone or SNAP and 20 μM ODQ plus L-Cys for 15 min, then either left unstimulated (Mock) or stimulated with ATP (ATP, 30 min) or nigericin (Nig, 30 min). **(B)** Immunoblot analysis of caspase-1 in lysates of peritoneal macrophages treated as in **A**. **(C)** Production of IL-1β by WT or iNOS$^{-/-}$ peritoneal macrophages primed with LPS for 24 h in the presence of medium (Mock) or 8-Br-cGMP (1 mM), and then stimulated with ATP. n.s., not significant. **(D)** Immunoblot analysis of caspase-1 and iNOS in lysates of WT or iNOS$^{-/-}$ peritoneal macrophages treated as in **C**. **(E)** Flow cytometry analysis of WT or iNOS$^{-/-}$ peritoneal macrophages stimulated with LPS for different lengths of time, left unstained or labeled with MitoSOX, and then stimulated with ATP. **(F)** Flow cytometry analysis of WT or iNOS$^{-/-}$ peritoneal macrophages left untreated (control) or stimulated with LPS for different lengths of time and then treated with ATP. The cells were stained for 15 min with MitoTracker Deep Red and MitoTracker Green before ATP treatment. **(G)** Production of IL-1β by WT or iNOS$^{-/-}$ peritoneal macrophages primed with LPS for 24 h and stimulated with ATP in the absence or presence of different concentrations of Mito-TEMPO. **(H)** Immunoblot analysis of caspase-1 and iNOS in lysates of WT or iNOS$^{-/-}$ peritoneal macrophages treated as in **G**. Actin serves as a loading control **(B**, **D** and **H)**. ProCasp1, procaspase-1; Casp1 p10, active form of caspase-1. ***$P < 0.001$ (Student's *t*-test). The data represent at least three experiments (mean ± SD in **A**, **C** and **G**).

NO exerted its effect by activating soluble guanylyl cyclase (sGC), thus elevating cGMP, a well-established downstream effector of NO [31]. To test this hypothesis, LPS-primed mouse peritoneal macrophages were stimulated with ATP or nigericin in the presence of NO and 20 μM ^{1}H-oxodiazolo-(1,2,4)-(4,3-a) guinoxaline-1-one (ODQ), a competitive inhibitor of sGC activation. ODQ did not reverse the inhibitory effect of NO on IL-1β production or caspase-1 activation (Figure 5A and 5B). Conversely, when the wild-type or iNOS-deficient peritoneal macrophages were stimulated with LPS for 24 h in the absence or presence of 1 mM 8-bromo-cGMP (8-Br-cGMP, an analog of cGMP) and then pulsed with ATP, there was no effect of 8-Br-cGMP on IL-1β production or caspase-1 activation (Figure 5C and 5D). Therefore, we concluded that the effect of NO on NLRP3 inflammasome activation does not rely on the sGC-cGMP signaling pathway.

Recent reports suggest that the mitochondria have a critical role in NLRP3 inflammasome activation [32, 33]. Therefore, we examined mitochondrial ROS production by measuring the fluorescence of MitoSOX, a mitochondrial superoxide indicator, in wild-type and iNOS-deficient macrophages stimulated with LPS for different lengths of time and then pulsed with ATP. After 6 h of LPS priming, treatment with ATP resulted in similarly increased mitochondrial ROS production in wild-type and iNOS-deficient cells. However, after 24 h of LPS priming, ATP-induced mitochondrial ROS production was only evident in the iNOS-deficient cells, whereas production in the wild-type cells was relatively low (Figure 5E). In addition, we assessed the functional mitochondria pool in wild-type and iNOS-deficient macrophages using MitoTracker Deep Red, a fluorescent probe sensitive to the mitochondrial inner transmembrane potential. Consistent with the pattern of mitochondrial ROS production, treatment with ATP resulted in a decreased percentage of intact mitochondria only after 6 h of priming in wild-type macrophages. In contrast, in iNOS-deficient cells, ATP treatment induced a large accumulation of damaged mitochondria after both 6 h and 24 h of LPS priming (Figure 5F). Furthermore, dysfunction of mitochondria resulted in the release of mitochondrial DNA (mtDNA) into the cytosol. Thus we examined the mtDNA in the cytosolic compartment. Consistent with mitochondrial ROS production patterns and the changes in mitochondrial transmembrane potential, iNOS deficiency led to increased mtDNA translocation to the cytosol (Supplementary information, Figure S5). Therefore, the disruption of iNOS generates a defect in mitochondrial homeostasis, resulting in more mitochondrial ROS production, mitochondrial damage and mtDNA release in response to ATP stimulation at the late stage of LPS priming.

To further investigate whether the excessive production of mitochondrial ROS was responsible for the enhanced IL-1β production and caspase-1 activation in iNOS-deficient macrophages, we primed wild-type and iNOS-deficient macrophages with LPS for 24 h and then pulsed them with ATP in the presence of the mitochondria-targeted antioxidant Mito-TEMPO, a scavenger specific for mitochondrial ROS, or total ROS scavenger N-acetyl-L-cysteine (NAC) or diphenyleneiodonium (DPI). As shown in Figure 5G and 5H, Mito-TEMPO abolished IL-1β secretion and caspase-1 activation in the iNOS-deficient macrophages. Both NAC and DPI had the similar effect as Mito-TEMPO (Supplementary information, Figure S6). These data demonstrate that the inhibition of the NLRP3 inflammasome by NO depends on mitochondrial ROS production.

Increased susceptibility to LPS-induced septic shock in NO-deficient mice

To evaluate the role of endogenous NO in the activation of the NLRP3 inflammasome *in vivo*, we tested the effect of NO in the mouse model of LPS-induced septic shock. Although research has been conducted on the function of NO during septic shock, the conclusions have been controversial due to the variety of experimental conditions adopted [34, 35]. In our experiments, IL-1β secretion was significantly increased in the peritoneal lavage fluid from mice treated with the NOS inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), and in iNOS-deficient mice compared with the control and wild-type mice after the peritoneal injection of LPS (Figure 6A and 6C). Furthermore, IL-18 production was enhanced in the sera of the iNOS-deficient mice (Figure 6C), and L-NAME treatment or iNOS deficiency led to much higher mortality rates (Figure 6B and 6D).

To determine whether the *in vivo* effect of NO was mediated by the NLRP3 inflammasome, we generated NLRP3-deficient mice (CMV-NLRP3 KO) by crossing NLRP3-R258W knock-in mice [36] with CMV-Cre mice (Supplementary information, Figure S7A). To verify the successful deletion of NLRP3 in the CMV-NLRP3 KO mice, we differentiated bone marrow-derived macrophages from the wild-type and CMV-NLRP3 KO mice and exposed these cells to NLRP3 stimuli. As expected, NLRP3 was successfully deleted in CMV-NLRP3 KO macrophages, whereas the expression of ASC was normal (Supplementary information, Figure S7C). As a result, neither IL-1β secretion nor caspase-1 activation was detected in the CMV-NLRP3 KO macrophages (Supplementary information, Figure S7B and S7C). In addition, the *in vivo* production of IL-1β and IL-18 was complete-

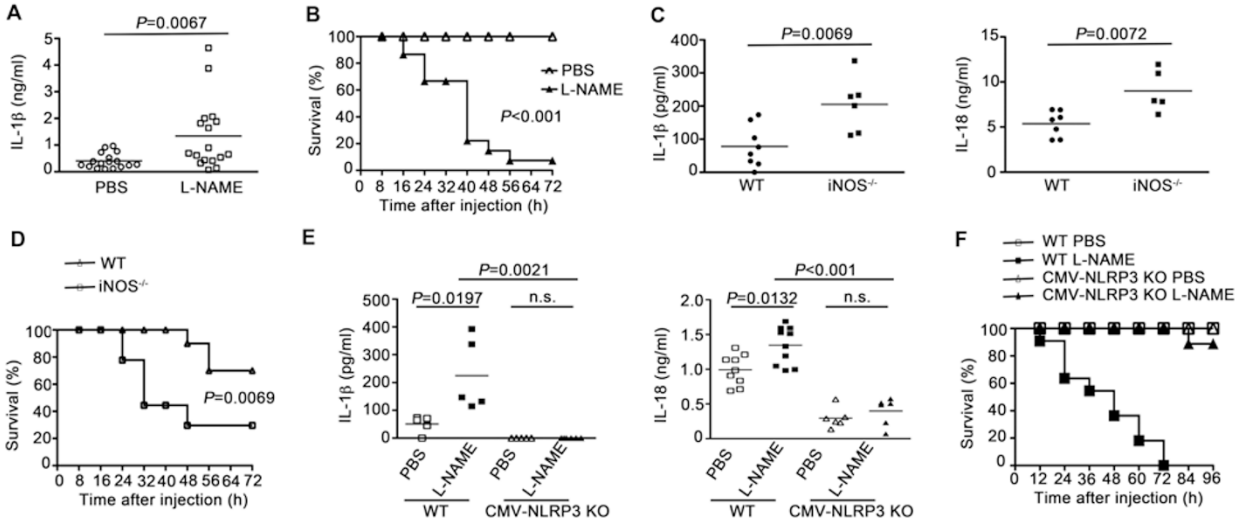

Figure 6 NOS inhibition and iNOS deficiency enhance *in vivo* IL-1β and IL-18 secretion, and increase susceptibility to LPS-induced death. **(A)** Production of IL-1β in peritoneal lavage fluid at 8 h after intraperitoneal injection of LPS (1.5 mg/kg of body weight) without (PBS, *n* = 17) or with (L-NAME, *n* = 17) NOS inhibitor L-NAME (100 mg/kg of body weight) into 7-week-old female C57BL/6 mice. **(B)** Survival of 7-week-old female C57BL/6 mice injected intraperitoneally with LPS (5 mg/kg of body weight) without (PBS, *n* = 14) or with (L-NAME, *n* = 14) NOS inhibitor L-NAME (100 mg/kg of body weight). Lethality was recorded for 96 h. **(C)** Production of IL-1β in peritoneal lavage fluid and IL-18 in serum 24 h after intraperitoneal injection of LPS (10 mg/kg of body weight) into female WT and iNOS$^{-/-}$ mice. **(D)** Survival of female WT (*n* = 10) and iNOS$^{-/-}$ (*n* = 9) mice injected intraperitoneally with LPS (10 mg/kg of body weight). Lethality was recorded for 96 h. **(E)** Production of serum IL-1β and IL-18 at 8 h after intraperitoneal injection of LPS (5 mg/kg of body weight) without (PBS) or with (L-NAME) NOS inhibitor L-NAME (100 mg/kg of body weight) into 7-week-old female WT and CMV-NLRP3 KO mice. n.s., not significant. **(F)** Survival of 7-week-old female C57BL/6 and CMV-NLRP3 KO mice (*n* = 9 per group) injected intraperitoneally with LPS (5 mg/kg of body weight) without (PBS) or with (L-NAME) NOS inhibitor L-NAME (100 mg/kg of body weight). Lethality was recorded for 96 h. The data are representative of two independent experiments.

ly eliminated in the CMV-NLRP3 KO mice after LPS injection (Supplementary information, Figure S7D). These results thus thoroughly confirmed the deletion of NLRP3 in the CMV-NLRP3 KO mice. We then challenged the wild-type or CMV-NLRP3 KO mice with LPS in the presence or absence of L-NAME. In wild-type mice, L-NAME treatment resulted in increased serum IL-1β and IL-18, whereas NLRP3 deficiency led to undetectable IL-1β , and dramatically decreased the IL-18 production regardless of treatment with L-NAME (Figure 6E). Notably, in sharp contrast to the reaction in the wild-type mice, L-NAME did not increase the susceptibility to LPS in the CMV-NLRP3 KO mice (Figure 6F). Thus, the NO produced by iNOS under inflammatory conditions attenuates disease development by inhibiting the activation of the NLRP3 inflammasome *in vivo*.

Discussion

In addition to its antibacterial effects, NO is known as an immunomodulatory molecule, although the mechanisms underlying this immunomodulation are poorly understood. Our work suggests that the anti-inflammatory effects of NO may result from its ability to suppress NLRP3 inflammasome activation, in turn inhibiting IL-1β secretion, caspase-1 cleavage and ASC pyroptosome formation. However, NO does not affect IL-6 or TNF-α production and NLRP3 or ASC expression. NO thus specifically inhibits NLRP3 inflammasome activation but not the TLR/NF-κB signaling pathways.

It has been reported that NO can inhibit caspase-1 activity by direct modification [29, 30]. In this study, we found that NO not only inhibits NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation, but also inhibits NLRC4- and AIM-2-mediated IL-1β secretion and caspase-1 activation (Supplementary information, Figure S8), but to a much less extent (3-fold decrease for NLRC4 and AIM-2, 40-fold decrease for NLRP3). However, NO suppresses NLRP3-mediated but not NLRC4- or AIM-2-mediated ASC pyroptosome formation (Supplementary information, Figure S9). These results demonstrate that aside from caspase-1, NO also affects upstream signaling of NLRP3, such as mitochondrial ROS production. As the formation of ASC pyropto-

some is independent of caspase-1, and NLRC4 and AIM-2 are activated independent of mitochondrial signaling [37], it is thus conceivable that NO has no effect on NLRC4- or AIM-2-mediated ASC pyroptosome formation. Thus, the inhibitory effect of NO on ASC pyroptosome formation is NLRP3 specific.

The NLRP3 inflammasome is activated in a two-step manner [38]. In the first step, LPS or another TLR agonist induces NLRP3 and pro-IL-1β expression through the NF-κB pathway. After this priming phase, the NLRP3 inflammasome can be activated by ATP, nigericin, MSU crystals or other stimuli. In the current study, we found that the NLRP3 inflammasome was activated in a time-dependent fashion during the priming stage. This phenomenon is inversely correlated with the expression of iNOS, a NOS induced by LPS. Using an iNOS inhibitor, iNOS-specific siRNA and iNOS-deficient macrophages, we found that the endogenous NO derived from iNOS negatively regulates NLRP3 inflammasome activation at the late stage of LPS priming. Furthermore, overexpression of iNOS can inhibit IL-1β production and caspase-1 activation (data not shown). These data demonstrate that endogenous NO functions as a negative modulator of the NLRP3 inflammasome. In a previous publication, we reported that another LPS-induced protein, Trim30, inhibits NLRP3 inflammasome activation [39]. Taken together, our findings indicate that at the late stage of LPS stimulation, the host cell can restrict the inflammatory response by inducing negative regulators such as iNOS and Trim30.

In this study, we find that NO donor not only inhibits NLRP3 inflammasome activation in mouse macrophages, but also suppresses human NLRP3 inflammasome activation in human THP-1 cells and primary PBMCs. Although it is difficult to induce endogenous NO in human monocytes [40], other cells such as hepatocytes are known to make large quantities of NO via iNOS [41, 42], which could act to inhibit NLRP3 inflammasome activation in monocytes through a paracrine mechanism.

Recently, two reports suggested that mitochondria are critical for the activation of the NLRP3 inflammasome [32, 33]. In the current study, we indeed found that at the late stage of LPS priming, treatment with ATP results in more damage to the mitochondria in iNOS-deficient than in wild-type macrophages, indicated by increased mitochondrial ROS production and the loss of mitochondrial integrity. Furthermore, treatment with the ROS scavenger Mito-TEMPO, NAC or DPI reverses the excessive IL-1β and IL-18 secretion and caspase-1 activation resulting from iNOS deficiency. These findings indicate that NO could be a critical regulator of mitochondrial function in macrophages.

NO is a reactive molecule that has a variety of effects. One of the main pathways in NO signaling is effected by sGC, an enzyme that converts GTP into the important intracellular signaling molecule cGMP. The NO-mediated inhibition of NLRP3 inflammasome activation appears to be independent of the classical sGC-cGMP pathway because physiological concentration of 8-Br-cGMP has no effect on NLRP3 inflammasome activation in iNOS-deficient macrophages. Moreover, ODQ, a specific inhibitor of sGC, does not reverse the inhibitory effect of NO either. In addition to inducing cGMP production, NO may exert its effects by inducing the formation of S-nitrosothiols from cysteine residues via S-nitrosylation and by modifying the activity of several proteins involved in cellular regulation [43]. However, we found that neither NLRP3 nor ASC is modified by NO through S-nitrosylation (data not shown). Alternatively, NO may maintain mitochondrial homeostasis by inducing the S-nitrosylation of mitochondrial proteins [44]. As the molecular mechanisms by which NLRP3 agonists induce mitochondrial ROS production and mitochondrial ROS activate the NLRP3 inflammasome are unknown, the direct target of NO should be investigated in future studies.

Over the past two decades, the function of NO in inflammatory responses, including septic shock, has been thoroughly investigated, yet the conclusions of these studies remain controversial. Using iNOS-deficient mice, at least three laboratories have studied the physiological function of NO during LPS-induced septic shock [45-48]. One study showed that iNOS-knockout mice had a significantly higher mortality rate than wild-type mice. However, others showed that iNOS-knockout mice had a lower mortality rate than wild-type mice [48]. The basis of these differences between the reports is unknown. In the current study, we found that the inhibition of endogenous NO by L-NAME or the deletion of iNOS increased the production of caspase-1-dependent cytokines and mortality in mice after LPS challenge. This finding is consistent with clinical reports showing that treatment with NOS inhibitors results in a higher mortality rate of septic patients [49]. However, we found that the administration of the NOS inhibitor L-NAME did not increase the mortality rate of NLRP3-deficient mice. These data further indicate that the anti-inflammatory effect of NO is mediated by the suppression of the NLRP3 inflammasome.

In conclusion, our study has provided a mechanistic explanation of how NO, a multi-functional molecule, modulates NLRP3 inflammasome-mediated immune responses. Intriguingly, our findings suggest that NO could be an intrinsic negative regulator of a range of diseases induced by abnormal NLRP3 inflammasome activation.

Our observation that the human NLRP3 inflammasome is inhibited by NO further suggests a potential therapeutic application of NO in the treatment of inflammatory diseases.

Materials and Methods

Mouse strains and reagents

C57BL/6, iNOS$^{-/-}$, caspase-1$^{-/-}$ and CMV-Cre transgenic mice were obtained from the Jackson Laboratory. The generation of NLRP3-R258W knock-in mice has been described [36]. To generate CMV-NLRP3 knockout mice, we crossed the NLRP3-R258W knock-in mice with CMV-Cre mice. All of the mice were maintained in specific pathogen-free (SPF) facilities at the Animal Care Facility of the Chinese Academy of Sciences. The animal care and use complied with institutional guidelines.

All of the reagents were from Sigma unless stated otherwise. Anti-NLRP3 antibody was obtained from Alexis (804-880-C100). Anticaspase-1 antibody was purchased from Santa Cruz Biotechnology. The MSU crystals were prepared as previously reported [6]. The IL-1β, TNF-α and IL-6 ELISA kits were obtained from R&D Systems, and the IL-18 ELISA kit was purchased from MBL International Corporation.

Cells

In preparation for the isolation of peritoneal macrophages, mice were intraperitoneally injected with 1 ml of 4% thioglycollate, and peritoneal exudate cells were isolated from the peritoneal cavity 4 days post injection. The cells were then incubated at 37 °C for 6 h and washed three times with HBSS. The remaining adherent cells were used as the peritoneal macrophages described in the experiments. Unless otherwise indicated, the macrophages were primed with 200 ng/ml LPS from *Escherichia coli* 0111:B4 (Sigma) for 4 h before stimulation with 5 mM ATP for 30 min, 20 μM nigericin for 30 min or 250 μg/ml MSU for 3 h. For pharmacological assessments, SNAP or GSNO was added to the cell culture 15 min before the stimulation with ATP, nigericin or MSU crystals.

The bone marrow-derived macrophages were prepared as follows: bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice, and subsequently depleted of red blood cells using ammonium chloride. The cells were then cultured at 2×10^6 cells per well in 24-well plates in DMEM supplemented with 20 ng/ml murine M-CSF. Nonadherent cells were carefully removed, and fresh medium was added every 2 days. On day 6, the cells were collected for experiments.

Human peripheral blood monocytes were purchased from the Shanghai Blood Center. The cells were adjusted to 5×10^6 cells/ml and resuspended in RPMI-1640 culture medium supplemented with 50 mg/ml gentamicin, 2 mM L-glutamine and 1 mM pyruvate.

siRNA synthesis and transfection

The control siRNA and iNOS-specific siRNAs were purchased from Invitrogen. The forward siRNA sequences targeting iNOS were as follows: S1, 5′-UUUCAAAGACCUCUGGAUCUUGACC-3′ and S2, 5′-AGAGUGAGCUGGUAGGUUCCUGUUG-3′. The siRNA was delivered into the macrophages using Lipofectamine 2000 Transfection Reagent (Invitrogen).

Retrovirus preparation and infection

Retrovirus was prepared as previously reported [50]. Briefly, iNOS was cloned into MSCV-IRES-GFP, and transfected together with pCMV/VSV-G and pKF3-RSV/Gag-Pol into 293T cells using Lipofectamine. After 24 h, the medium was replaced with DMEM containing 10% FCS. Forty-eight hours post transfection, the retroviral supernatants were harvested, supplemented with 6 μg/ml polybrene, and used to infect bone marrow cells. The cell culture plates were then centrifuged at 1 200× *g* for 90 min at room temperature. After 12 h of incubation at 37 °C, the retroviruses were removed, and the cells were cultured in fresh medium.

Confocal microscopy

Macrophages were plated overnight on coverslips, stimulated as described above and stained with antiASC antibody, anticaspase-1 antibody and DAPI. After stimulation, the cells were washed and fixed with 4% PFA in PBS for 15 min, blocked with 10% FBS in PBS and permeabilized with Triton X-100. The cells were then incubated with FITC-conjugated antiASC antibody for 1 h and rinsed with PBST. Finally, the cells were stained with DAPI. The confocal microscopy analyses were performed using a Leica TCS SP2.

ASC pyroptosome detection

ASC pyroptosomes were detected as previously reported [26]. Macrophages were seeded in 6-well plates (2×10^6 cells per well) and treated with different stimuli. The cells were pelleted by centrifugation and resuspended in 0.5 ml of ice-cold buffer containing 20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1% Nonidet P-40, 0.1 mM PMSF and a protease inhibitor mixture, and lysed by shearing 10 times through a 21-gauge needle. The cell lysates were then centrifuged at 5 000× *g* for 10 min at 4 °C, and the resultant pellets were washed twice with PBS and resuspended in 500 μl of PBS. Next, the resuspended pellets were crosslinked with fresh DSS (4 mM) for 30 min and pelleted by centrifugation at 5 000× *g* for 10 min. The crosslinked pellets were resuspended in 30 μl of SDS sample buffer, separated using 12% SDS-PAGE and immunoblotted using anti-mouse ASC antibodies.

Flow cytometry

Mitochondrial ROS were measured in cells by MitoSOX (Invitrogen) staining (5 μM for 15 min at 37 °C). The cells were then washed with PBS and stimulated with ATP for 30 min. To measure the mitochondrial mass, the cells were stained for 15 min at 37 °C with 25 nM MitoTracker Green FM and MitoTracker Deep Red FM (Invitrogen), followed by 30 min of ATP treatment. After stimulation, the cells were harvested and resuspended in PBS solution containing 1% FBS for FACS analysis. The data were acquired with a FACSCanto II (BD Biosciences) and analyzed with FlowJo analytical software (TreeStar).

Immunoblot analysis

For the immunoblot analysis, whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad) and hybridized with various primary antibodies. After incubation with HRP-labeled antimouse IgG (HAF 007, R&D Systems) or antirabbit IgG (4050-05, Southern Biotechnology Associates), the proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). For caspase-1 p10 detection, the blots

were incubated with rabbit polyclonal antibody against mouse caspase-1 p10 (sc-514, Santa Cruz Biotechnology) and visualized with SuperSignal West Dura Extended Duration Substrate (34075, Pierce).

ELISA

Mouse and human cytokines in culture supernatants were measured with ELISA kits. Mouse IL-18 in plasma was also measured by ELISA.

In vivo septic shock model

To induce *in vivo* cytokine secretion, 7-week-old female mice were injected intraperitoneally with LPS. Six hours after the injection, the peritoneal cavities were washed with 0.8 ml PBS containing 1% FBS. IL-1β in the peritoneal lavage fluids and IL-18 in the sera were then measured by ELISA. To induce septic shock, the mice were injected intraperitoneally with LPS, and their health status was monitored at regular intervals.

Statistical analysis

The data are presented as the mean ± SD of three independent experiments unless otherwise noted. The statistical comparisons between the different treatments were performed using an unpaired Student's *t*-test in which $P < 0.01$ was considered significant and $P < 0.001$ was highly significant.

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