Priming of NLRP3 inflammasome activation 1 by Msn Kinase MINK1 in Macrophages

Priming of NLRP3 inflammasome activation by Msn Kinase MINK1 in Macrophages

Kaixiang Zhu[1,2,7,11], Xuexiao Jin[1,11], Zhexu Chi[3], Sheng Chen[3,4], Songquan Wu[5], Richard D Sloan[2,6], Xuai Lin[7], Dante Neculai[8], Di Wang[3], Hu Hu[9], Linrong Lu[1,2,10, *]

[1]Institute of Immunology, and Department of Rheumatology in Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310058, P. R. China;

[2]Zhejiang University–University of Edinburgh Institute, Zhejiang University School of Medicine, 314400 Haining, P. R. China;

[3]Department of Immunology, Zhejiang University School of Medicine, Hangzhou 310058, P. R. China;

[4]Department of Colorectal Surgery of the Second Affiliated Hospital, Hangzhou 310058, P. R. China;

[5]Medical College of Lishui University, Lishui, 323000, P. R. China;

[6]Infection Medicine, School of Biomedical Sciences, The University of Edinburgh, Edinburgh EH16 4SB, Scotland, UK;

[7]Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou 310058, P. R. China;

[8]Department of Cell Biology, Zhejiang University School of Medicine, Hangzhou, 310058, P. R. China;

[9]Department of Pathology and Pathophysiology, Zhejiang University School of

Medicine, Hangzhou 310058, P. R. China;

[10]Dr. Li Dak Sum and Yip Yio Chin Center for Stem Cells and Regenerative Medicine, Zhejiang University School of Medicine, Hangzhou, 310058, P. R. China;

*To whom correspondence should be addressed:
Linrong Lu, 866 Yuhang Tang Road, Medical Research Building A807, Hangzhou, 310058, P. R. China. Tel: 86-571-88981173; Email: lu_linrong@zju.edu.cn.

[11]These authors contributed equally

Running title: MINK1 kinase pre-set NLRP3 inflammasome activation through direct phosphorylation.

Abstract

The NLRP3 (nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain-containing 3) inflammasome is essential in inflammation and inflammatory disorders. Activation of the inflammasome is differentially regulated by phosphorylation at various sites on NLRP3. Ser 725 is a phosphorylation site on NLRP3 depicted in multiple inflammasome activation scenarios, yet the importance as well as the regulation of this site has not been clarified. In this study, we reveal that the phosphorylation of Ser 725 is an essential step for the priming of NLRP3 inflammasome in macrophages. We also show that Ser 725 is directly phosphorylated by Misshapen (Msn) / NIK-related kinase 1 (MINK1), depending on the direct interaction between MINK1 and NLRP3 LRR domain. MINK1 deficiency leads to reduced NLRP3 activation and dampened inflammatory responses in mouse models of acute sepsis and peritonitis. Moreover, reactive oxygen species (ROS) upregulate the kinase activity of MINK1 and subsequently promote inflammasome priming through NLRP3 Ser 725 phosphorylation. Eliminating ROS suppressed NLRP3 activation and reduced both sepsis and peritonitis symptoms in a MINK1 dependent manner. Altogether, our study unveils a direct regulation of the NLRP3 inflammasome by Msn family kinase MINK1 and suggests that modulating the kinase activity of MINK1 could be a potential intervention strategy for inflammasome-related diseases.

Keywords: MINK1 kinase, NLRP3 inflammasome, ROS, phosphorylation.

Introduction

The NLRP3 inflammasome plays a key role in host defense against pathogens(1, 2). NLRP3 can sense a variety of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) during infection and tissue damage. It forms a cytosolic multimeric protein complex to process the maturation of caspase-1 and the release of several pro-inflammatory cytokines including interleukin-1β (IL-1β) and IL-18(3-5). On the other hand, dysregulated activation of the NLRP3 inflammasome is closely related to various inflammatory disorders and inflammation-related diseases including sepsis, type-2 diabetes (T2D), gout and acute peritonitis (1, 4, 6-10). Classic NLRP3 inflammasome activation follows a 2-step model, requiring both priming and assembling(11). The priming step is instructed by PAMPs or cytokines like IL-1β and TNF, and is essential to upregulate the transcription levels of NLRP3 and other inflammasome components(12). The subsequent activation step is driven by numerous PAMPs or DAMPs, and involves the activation of multiple upstream signaling events (4, 13).

NLRP3 activation is extensively regulated by phosphorylation and can be phosphorylated at different inflammasome phases, including the unstimulated, priming, activation and resolution phases. Song et al. demonstrated that JNK1-mediated NLRP3 phosphorylation at Ser 194 residue is a key molecular priming event that poises NLRP3 for self-association and inflammasome assembly (14). Interestingly, it has been shown that phosphorylation of NLRP3 can either activate or inhibit the activation of NLRP3 (14-18). Previous research showed that Phosphatase 2A (PP2A) can license inflammasome assembly via dephosphorylating NLRP3 pyrin domain (PYD), while phosphatase and tensin homolog deleted on chromosome ten (PTEN) can directly interact with and dephosphorylate NLRP3 to enable NLRP3–ASC (apoptosis-associated speck-like protein containing a CARD) interaction, inflammasome assembly and activation (17, 19). Thus, in-depth exploration of the roles of phosphorylation and the responsible kinases in the regulation of NLRP3 inflammasome activation may lead

us to find out the therapeutic targets of NLRP3 inflammasome and therefore treat NLRP3-related inflammatory diseases. According to the protein phosphorylation site database PhosphoSitePlus (phosphosite.org), Ser 725 (the locus corresponding to human NLRP3 S728) is one of the most highly phosphorylated sites in mouse NLRP3, yet its function as well as the kinase responsible for this site has not been uncovered (14, 17).

Misshapen (Msn) / NIK-related kinase 1 (MINK1) is a serine-threonine kinase protein belonging to the GCK protein family (20-22). MINK1 was initially described as a signaling mediator of the MAP kinase cascades, thus also named MAP4 kinase 6 (MAP4K6) (20). It was later found to play a role in mediating Ras-induced senescence downstream of reactive oxygen species (ROS) in ovarian epithelial cells (23). Additionally, MINK1 was reported to be involved in various biological process such as neurodegeneration, thymocyte selection, and platelet function (24-27). Moreover, MINK1 kinases can act as a kinase in the Hippo pathway and activate LATS1/2 in parallel to MST1/2, which affects tumor immunity (28, 29). Our recent research has revealed the contribution of MINK1 in regulating Th17 cells differentiation (30). We found that the knockout of MINK1 in mice resulted in Th17 cell accumulation and increased susceptibility to EAE. However, dysregulated activation of T cells did not result in spontaneous inflammatory symptoms in MINK1 knockout mice, suggesting additional roles of MINK1 in other cell types.

Given the fact that MINK1 expression is highly enriched in myeloid cells, but to a lesser extent in dendritic cells (DCs), we have gone on to study its function in macrophages. We now report that MINK1 deficiency dampened NLRP3 inflammasome activation and IL-1β secretion in macrophages. We demonstrate that MINK1 can be activated by ROS and promote NLRP3 activation through direct phosphorylation of the Ser 725 residue in the NLRP3 LRR domain, which serves as an essential priming event for NLRP3 self-interaction and oligomerization. *In vivo*, disruption of MINK1 kinase activity by ROS scavengers markedly alleviated the activation of NLRP3 and improved the pathogenesis of inflammatory diseases. Our research thus not only reveals a new

regulatory function of MINK1 kinase in macrophages, but also provides a previously unknown mechanistic explanation for how ROS activate the NLRP3 inflammasome.

Results

MINK1 promotes NLRP3-dependent caspase-1 maturation and IL-1β production. To assess the possible involvement of MINK1 kinase in NLRP3 inflammasome activation, we treated LPS-primed bone marrow-derived macrophages (BMDMs) from WT and *Mink1*$^{-/-}$ mice with different agonists. *Mink1*$^{-/-}$ BMDMs showed impaired NLRP3 inflammasome activation when triggered by ATP, nigericin and Alum compared to that of WT mice, which was reflected by reduced levels of IL-1β cleavage and caspase-1 maturation (Fig. 1A), and lower IL-1β and IL-18 secretion (Fig.1B). The reduction of IL-1β secretion in *Mink1*$^{-/-}$ BMDMs was observed under both optimal and suboptimal ATP concentrations (1mM to 3mM) (Fig. S1A). Moreover, NLRP3-dependent ASC oligomerization and ASC speck formation were also decreased in *Mink1*$^{-/-}$ BMDMs (Fig 1E and F). These results suggest a requirement of MINK1 for NLRP3 inflammasome activation. Importantly, LPS induced expression of pro-IL-1β and pro-caspase-1, as well as secretion of tumor necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6) were not changed under different stimulation conditions (Fig. 1A and C, Fig. S1B, C, and D). We further investigated the role of MINK1 during the activation of other inflammasomes. We found that the activation of AIM2 or NLRC4, triggered by poly (dA:dT) transfection and *Salmonella Typhimurium* infection respectively, were not affected by MINK1 deficiency, indicating the specific function of MINK1 in NLRP3 activation (Fig. 1C and D, Fig. S1E). Together, these results demonstrate the essential role of MINK1 kinase in promoting NLRP3 inflammasome activation in macrophages.

MINK1 ablation ameliorates acute NLRP3 dependent inflammation

We next used LPS-induced sepsis and Alum-induced peritonitis models to evaluate the contribution of MINK1 towards the NLRP3-mediated inflammatory response *in vivo*. In the sepsis model, we observed a significant decline of serum IL-1β and IL-18 levels in *Mink1*$^{-/-}$ mice compared to WT mice, while the production of TNF-α and IL-6

remained comparable (Fig. 2A). In parallel, after the injection of LPS, the survival rate of *Mink1*$^{-/-}$ mice was substantially improved relative to wild type (Fig. 2B). We next performed the fluorescent activity-based probe FLICA (FAM-7YVAD-FMK) staining to detect caspase-1 activation. Decreased activation of caspase-1 in peritoneal macrophages, spleen macrophages, and PBMCs from *Mink1*$^{-/-}$ mice was observed compared to those from the WT mice (Fig. 2C and D). Further statistical analysis confirmed the reduced frequency as well as reduced fluorescence intensity of FLICA^{+} macrophages in the *Mink1*$^{-/-}$ mice (Fig. S2A and B). After LPS injection, *Mink1*$^{-/-}$ mice also showed lower inflammatory cell infiltration, edema, interstitial hyperemia, hemorrhage, and alveolar collapse tissue damage in the lung (Fig. 2E, right). Similar observations were made in the liver tissue (Fig. 2E, left). In the Alum-induced acute peritonitis experiment, the *Mink1*$^{-/-}$ mice also showed milder inflammatory symptoms than control mice, evidenced by decreased numbers of neutrophils as well as decreased IL-1β level in the peritoneal fluid (Fig. 2F and G), although the frequency of neutrophils (CD45^{+} CD11b^{+} Gr-1^{+}) and TNF-α release in the peritoneal flush fluid showed no difference between the WT and *Mink1*$^{-/-}$ mice (Fig. 2G). Taken together, our results demonstrate that MINK1 kinase promotes NLRP3-mediated acute inflammation responses.

MINK1 relies on its kinase activity to promote NLRP3 activation.

We next investigated the mechanism of how MINK1 promotes the activation of the NLRP3 inflammasome. We first checked the signaling pathways essential for priming NLRP3 inflammasome activation (31-33). No significant difference was found in the upstream events of NLRP3 activation in BMDMs from *Mink1*$^{-/-}$ and WT mice (Fig. S3A). RT-qPCR analyses also showed that the RNA expression levels of the NLRP3 inflammasome components remained unaffected upon MINK1 deficiency (Fig. S1F). Taken together the fact that the expression of pro-caspase-1 and pro-IL-1β were also unaltered (Fig. 1A and C), we concluded that the regulation of NLRP3 activation by MINK1 was independent of its effect on the expression of essential NLRP3 components

in macrophages.

MINK1 is a member of the mammalian GCK family of kinases, whose multiple functions are highly dependent on its kinase activity (34, 35). Given that NLRP3 activation is regulated by protein phosphorylation (19, 36), we next investigated whether the kinase activity of MINK1 is essential for promoting NLRP3-inflammasome activation. MINK1 is composed of an N-terminal catalytic domain (kinase domain), a C-terminal citron homology (CNH) domain (regulatory domain), and an intermediate region (Fig. 3A and (34)). We reconstituted *Mink1*$^{-/-}$ BMDMs with the MINK1 kinase domain and regulatory domain respectively. We found that the overexpression of the kinase domain, but not the regulatory domain, could restore ATP-induced IL-1β secretion and caspase-1 activation (Fig. 3B and C), while TNF-α release was not affected (Fig. 3D). To further corroborate this, we reconstituted the NLRP3 inflammasome in HEK293T cells with WT MINK1 and a MINK1^{K54R} mutant which abrogates the MINK1 kinase activity. We found that the inactive MINK1^{K54R} mutant was unable to induce caspase-1 maturation and IL-1β secretion compared to the WT protein (Fig. 3E and F, Fig. S3B). Collectively, these results suggest that the kinase activity of MINK1 is required for NLRP3-inflammasome activation.

MINK1 directly interacts with NLRP3.

We next investigated whether MINK1 interacts directly with the key components of the NLRP3 inflammasome. In immunoprecipitation (IP) assays for BMDMs and PMs, we found that ATP stimulation could promote the specific binding of endogenous MINK1 kinase to NLRP3 but not to the other components including ASC, pro-caspase-1, pro-IL-1β or NEK7 (Fig. 3G, Fig. S3D-G). Immunofluorescence results also revealed substantial co-localization of MINK1 and NLRP3 (Fig. 3H and Fig. S3C). When co-expressed in HEK293T cells, the interaction of MINK1 kinase and NLRP3 was also found (Fig. 3I). We were then able to further map the physical domains responsible for MINK1-NLRP3 interaction in HEK293T cells. We found that the MINK1 kinase domain could directly bind to NLRP3, while mutant MINK1^{K54R} (which has ablated

kinase activity) lost the ability to bind to NLRP3 (Fig. 3J and K). These results further confirmed the function of MINK1 kinase and its kinase activity in NLRP3 inflammasome activation. When different NLRP3 truncated mutants were expressed with MINK1 in HEK293T cells, we found that only the LRR domain of NLRP3 was capable of binding with MINK1 (Fig. 3L). These results suggest that MINK1 promotes NLRP3 inflammasome activation through direct interaction with NLRP3.

MINK1-mediated NLRP3 phosphorylation is essential for inflammasome activation.

To determine whether MINK1 could phosphorylate NLRP3 directly, we used Phos-tag^{TM} SDS-PAGE to detect the phosphorylation of NLRP3. Significantly decreased phosphorylation of NLRP3 was observed in *Mink1*$^{-/-}$ BMDMs (Fig. 4A and S4A). We also performed mass spectrometry (MS) analysis of NLRP3 in MINK1 overexpressing cells, which lead to the detection of Ser 725 phosphorylation (Fig. 4B and Fig. S4B). To further investigate how the phosphorylation of this residue could affect NLRP3 activation, we reconstituted the NLRP3 inflammasome in HEK293T cells with WT NLRP3, an NLRP3^{S725A} mutant (to mimic dephosphorylation), or the NLRP3^{S725D} and NLRP3^{S725E} mutants (to mimic phosphorylation) respectively. We found that ATP-induced caspase-1 activation and IL-1β production were abrogated in NLRP3^{S725A} cells, while remained intact in cells that were reconstituted with WT, NLRP3^{S725D} or NLRP3^{S725E} (Fig. 4C and D). When these mutants were overexpressed in BMDMs from *Nlrp3*$^{-/-}$ mice, NLRP3^{S725D} and NLRP3^{S725E} could restore ATP induced IL-1β and IL-18 secretion and caspase-1 activation to the level of WT cells, while NLRP3^{S725A} failed to do so (Fig. 4E and F). As expected, the production of TNF-α was not affected by any of these manipulations (Fig. 4E). These results suggest that MINK1 mediated phosphorylation at Ser 725 is essential for NLRP3 activation.

MINK1 promotes NLRP3 self-association.

NLRP3 self-association is a critical step in inflammasome assembly and activation (14).

We therefore investigated whether phosphorylation at the S725 site of NLRP3 mediated by MINK1 affects the assembly of the NLRP3 inflammasome. Flag-tagged NLRP3, with or without S725 mutation, was co-transfected with HA-tagged NLRP3 in HEK293T cells. We found the NLRP3 self-interaction was limited by the S725A mutation, while significantly enhanced by the S725D and S725E mutations (Fig. 4G). Similarly, reduction of NLRP3 self-interaction was also observed in cells overexpressing the kinase-dead MINK1 protein (Fig. 4H and Fig. S4D). To investigate potential structural mechanisms underlying phosphorylation-induced changes in NLRP3, we performed symmetric docking of human NLRP3 and NEK7 protein subunits. As this site is well conserved in different organisms (Fig. S4C), we used the structure of human NLRP3 in the database as a template. The calculated score of the complex of S728A (dephosphorylation-mimicking) mutant was significantly decreased compared with WT (Figure 4I, J and K), while S728E showed no significant difference (Fig S4E), which suggests that the dephosphorylation-mimicking mutant complex is less stable than the WT. These results revealed a regulatory role for S725 phosphorylation in NLRP3-NLRP3 interactions.

ROS activate MINK1 kinase to prime the activation of NLRP3

It has been reported that MINK1 can be activated by ROS, while our previous study showed that the kinase activity of MINK1 was profoundly reduced by NAC (ROS scavenger) treatment (23, 37). Furthermore, ROS can also serve as a priming factor for NLRP3 inflammasome (38, 39). We next asked whether MINK1-mediated NLRP3 phosphorylation and activation is a downstream event following ROS production. We found that the levels of total ROS and mitochondrial ROS production in LPS-primed BMDMs stimulated with ATP were comparable between *Mink1*$^{-/-}$ and the control (Fig. 5A), indicating that MINK1 ablation does not affect mitochondrial damage in NLRP3 activation. We next treated BMDM cells with ROS scavenger NAC or Mito-TEMPO (a mitochondria-targeted superoxide dismutase mimetic with superoxide and alkyl radical scavenging properties) under different stimulation (30, 40, 41). We found that

the IL-1β and caspase-1 maturation, as well as IL-1β secretion, were critically compromised by these treatments (Fig. 5B and C, Fig. S5B-E), while TNF-α release was not affected upon NAC or Mito-TEMPO treatment (Fig. S5A and E). More importantly, NAC or Mito-TEMPO treatment failed to further suppress IL-1β and caspase-1 maturation in *Mink1*$^{-/-}$ cells (Fig. 5B and C, Fig. S5B-E). In *in vitro* kinase activity assays, we further confirmed that the activation of MINK1 is a direct priming event after LPS treatment, which could again be significantly reduced by the removal of ROS (Fig. 5D and F), in accordance with the suppressed IL-1β and caspase-1 maturation and IL-1β secretion after treatment with NAC or Mito-TEMPO (Fig. 5D and E, Fig. S5F and G). These results together indicate that ROS scavengers could reduce NLRP3 inflammasome activation *in vitro* in a MINK1-dependent manner at the priming stage.

NAC alleviates NLRP3-mediated acute inflammatory response in both mouse model and human cells.

To determine whether reduced NLRP3 inflammasome activation upon NAC treatment could result in pathological improvements in acute inflammation, we again performed *in vivo* LPS-induced sepsis, with or without NAC treatment at different time points. We found that serum levels of IL-1β and IL-18 were consistently lower in NAC treated groups, although the reduction rates varied between groups (Fig. 6A, B and C). At the same time, the secretion of TNF-α and IL-6 were similar (Fig. S6A and B). Prophylactic treatment of NAC showed the most beneficial effects, while NAC treatment afterward was the least effective. FLICA staining assay also revealed blunted LPS-induced caspase-1 activation in macrophages from NAC treated mice (Fig. 6D). Additionally, the survival of mice challenged with LPS was significantly improved by NAC supplementation as well (Fig. 6F). These results confirmed the protective role of NAC supplementation in acute inflammation. More importantly, we found that NAC treatment in *Mink1*$^{-/-}$ mice only led to limited improvements of the inflammatory symptoms as evaluated by survival rate and the proportion of FLICA^{+} macrophages,

304 which was consistent with our *in vitro* results (Fig. 6E and F). These results again prove
305 that ROS is the major upstream activator of MINK1 in promoting NLRP3
306 inflammasome activation.
307 Finally, we examined whether similar mechanisms exist in humans. We performed
308 similar treatments in peripheral blood mononuclear cells (PBMCs) freshly isolated
309 from healthy donors. The results showed that both NAC and Mito-TEMPO suppressed
310 IL-1β production in a dose-dependent manner (Fig. 6G and H). In addition, when we
311 used siRNA to knockdown MINK1 in the human THP1 cell line (Fig. S6C), we found
312 that ROS scavenging reduced NLRP3 activation only in the control group, but not in
313 MINK1 knockdown cells (Fig. 6I). Taken together, these results indicate that MINK1
314 in macrophages contributes to the progression of NLRP3-dependent inflammation in
315 both mice and humans, suggesting MINK1 is a potential target for treating NLRP3-
316 related inflammatory diseases in human patients.
317

Discussion

The phosphorylation of NLRP3 in different residues has been revealed to contribute to NLRP3 activation (14, 17, 19). However, there are still contradictions between these findings and the mechanisms. In this study, we found that the phosphorylation of NLRP3 Ser 725 is a critical priming step in addition to its upregulated expression for inflammasome activation. This single amino acid phosphorylation in the LRR domain of NLRP3 facilitates its self-association. We also demonstrated that MINK1 kinase, whose activity is triggered by ROS upon LPS stimulation, is responsible for mediating this phosphorylation. Thus, we revealed an as yet undiscovered regulatory pathway associated with NLRP3 priming.

In human macrophage studies, it has been previously reported that the NLRP3 S728 (equivalent to murine NLRP3 S725 residue) is a phosphorylation site. But studies from Song and Stutz. et al considered phosphorylation of Ser 728 was not essential for human NLRP3 activation (14, 17), which is in contradiction to our findings. In order to clarify this, we also experimented with human macrophage cells and confirmed that S728 mutation in human NLRP3 impairs inflammasome activation. We think that the possible reason for this inconsistency may lay in the different experimental settings. However, it is worth noting that a non-phosphorylation mutation at the S728 residue did lead to reduced NLRP3 activation in these previous reports, although not as significant as what we observed here (14, 17). Thus, the importance of this residue, as well as its phosphorylation, needs to be re-evaluated.

We have previously shown that MINK1 knockout in mice leads to elevated Th17 cell polarization and increased susceptibility to EAE (30). However, we did not observe spontaneous autoimmune phenotype in the MINK1 deficient mice. Since MINK1 is widely expressed in different immune cells, it is thus possible that it may exert immune regulatory roles in other immune cells. We went on to investigate its possible role in the macrophages since macrophage is a major cell type in mediating inflammatory responses. This led to the finding of a regulatory role of MINK1 in NLRP3 priming and

inflammasome activation. It is thus likely that, in the MINK1 KO mice, insufficient inflammasome activation may counterbalance the pro-inflammatory effect of increased Th17 differentiation and leave the mice free of spontaneous inflammation.

Many infectious and stress signals can activate the NLRP3 inflammasome to elicit inflammatory responses. Reactive oxygen species (ROS) have been considered as a major intermediate trigger to activate the NLRP3 inflammasome in both the priming and activation stages (42, 43). Their function in NLRP3 activation is very well confirmed, as both ROS eliminating and blocking of cellular anti-oxidant responses can inhibit NLRP3 inflammasome activation (12, 44-46). However, the mechanisms or pathways responsible for this process are still elusive. Thioredoxin-interacting protein (TXNIP) and the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which are the two major responders involved in the response to oxidative stress, have been considered as the linkers between ROS and NLRP3 activation. Zhou et.al have proposed a model that TXNIP released from TRX during oxidative stress could bind to NLRP3 directly to activate the inflammasome (47). Park et al. demonstrated that TXNIP promoted activation of inflammasome indirectly; TXNIP can inhibit the transcriptional activity of NF-κB to attenuate the LPS-induced upregulation of inducible nitric oxide synthase (iNOS) (48). However, these mechanisms are not supported by the genetic evidence, since the wild type and *Txnip*$^{-/-}$ macrophages secreted similar levels of IL-1β upon different stimuli (49). The transcription factor Nrf2 is essential for antioxidant responses. Previous studies have demonstrated that macrophages from *Nrf2*$^{-/-}$ mice secreted less IL-1β than wild-type cells in response to NLRP3 activators, and several different mechanisms have been proposed. Nrf2 is in a complex comprising Keap1 and the mitochondrial outer-membrane protein PGAM5 in its resting state. When ROS cause dissociation of Nrf2 from the complex, some of them can translocate to the cytosol and interact with ASC to activate the inflammasome (50). Some Nrf2 translocate to the nucleus and upregulate heme oxygenase-1 (HO-1) expression, which in turn activates the inflammasome at the priming stage (51). Nrf2 can also upregulate the expression of anti-oxidant genes and downregulate the

expression of TXNIP to mediate the activation of NLRP3 (42). However, Nrf2 did not appear to have a direct role in priming, and all the above proposed mechanisms are indirect processes that involve transcriptional regulation(52). Consistent with previous findings that ROS could activate the kinase activity of MINK1, our findings here offered a more attractive mechanism that PAMP and DAMP-induced ROS can activate MINK1 to prime NLRP3 activation through direct phosphorylation. This post-translational mechanism could be fast and efficient, which is consistent with the general characteristics of rapid NLRP3 inflammasome activation.

Abnormal activation of NLRP3 inflammasome is closely related to many human diseases (1, 4, 16). Since NLRP3 activation and IL-1β accumulation could mediate inflammation during viral infection in lung and pathogenesis of ARDS, such as in severe COVID-19 (53), it seemed that targeting NLRP3 has great potential in treating these diseases. The definition of MINK1 function in inflammasome activation as we have shown here has added MINK1 as a promising candidate in the strategy of treatment of NLRP3-related diseases since targeting kinases has proven to be a highly practical approach.

Materials and methods

Mice

Mink1$^{-/-}$ mice (on a C57BL/6J background) were generated as described previously (27, 30). *Nlrp3*$^{-/-}$ mice (on a C57BL/6J background) were kindly provided by Prof. Di Wang (Zhejiang University). C57BL/6 (B6) mice were purchased from the Model Animal Research Center of Nanjing University. All mice were housed in a specific pathogen-free facility in the Laboratory Animal Center of Zhejiang University in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. The animal experimental protocols were approved by the Review Committee of Zhejiang University School of Medicine (Approval No. ZJU20210015) and were in compliance with institutional guidelines (The mice for the experiment were aged 6-8 weeks and weighed 20-25 g).

Human samples

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from 6 healthy volunteers aged 20–30 years (3 men and 3 women) who had given informed consent. In brief, human peripheral blood samples were centrifuged and the serum was discarded, then resuspend with PBS and added into the Lympholyte-H (Cedarlane). The mesospheric cells were taken and spread into 6-well plate after centrifugation. Cells were treated after 24 hours. To use these human materials for research purposes, prior written informed consent was given by the donors and approval was obtained from the Zhejiang University School of Medicine (Approval No. 2021-076).

Cells

Mouse bone marrow cells (BMDMs) were flushed from tibias and femurs with chilled Dulbecco's modified Eagle's medium (DMEM) and then cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 ng/ml macrophage colony stimulating factor (PeproTech) to generate BMDMs. Mouse peritoneal macrophages (PMs) were harvested 4 days after thioglycolate (Merck) injection. Stimulation conditions were as described previously(16). Briefly, cells were

primed with LPS (500 ng/mL) for 3.5 h in Opti-MEM, and followed by stimulation with various NLRP3 inflammasome activators respectively as indicated, [2 mM ATP (30 min); 10 μM nigericin (30 min); 200 μg/mL MSU (5 h); 300 μg/mL aluminum salts (5 h)]. For AIM2 inflammasome activation, poly(dA:dT) (1 μg/mL) was transfected using Lipofectamine 2000 (3 ml/mg DNA) (Invitrogen). For NLRC4 inflammasome activation, S. Typhimurium was grown overnight in Luria-Bertani broth, and BMDMs were infected for 1 h with the salmonella culture (1:100) and then incubated for another 1 h in the presence of gentamycin. For reconstruction of NLRP3 inflammasome in HEK293T cells, cells were seeded into 6 well plate at 5×10^5 per well in complete cell culture medium. After 24 hours, the cells were transfected with plasmids expressing pro-IL-1β (1μg), Myc-pro-caspase-1 (60ng), HA-ASC (150ng), HA-NLRP3 (200ng), and MINK1 or its mutants (200ng) using Lipofectamine 2000. 48 hours later, the cells were stimulated with NLRP3 inflammasome activators, cell lysates and precipitated supernatants were further analyzed by western blotting and ELISA

Reagents

Primary antibodies against NLRP3 (AdipoGen), mouse IL-1β (R&D Systems), mouse caspase-1 (p20) (AdipoGen), NEK7 (Santa Cruz Biotechnology), ASC (Santa Cruz Biotechnology), MINK1 (NOVUS) were used for western blotting. For stimulation, MSU, Nigericin, ATP and poly (dA:dT) were purchased from Sigma-Aldrich, LPS, MitoTracker and MitoSOX were obtained from Invitrogen. The Salmonella was a gift from Prof. Di Wang. The FAM-FLICA-caspase-1 assay kit was obtained from Immunochemistry. The cytoTox 96 Non-radioactive cytotoxicity assay kit for LDH release was obtained from Promega. Phos-tag^{TM} SDS-PAGE was obtained from Wako.

Protein precipitation from supernatant

Briefly, Supernatants from different stimulation conditions were collected and centrifuge to remove the precipitate, then 1/4 volume of chloroform and 1 volume of methanol were added to the supernatant. Spin at room temperature, 12000 rpm for 5 min. Aspirated the methanol layer and add 1 volume of methanol again. Spin at room temperature, 12000 rpm for 5 min again. Air dry or dry on heat block at 50 °C for 5

min. The dried samples were lysed in cell lysis buffer.

Flow cytometry and cell sorting

The antibodies and reagents were used for surface staining: Fixable Viability Dye, anti-CD45 (APC-Cyanine7, 30-F11), anti-CD11b (PE, N418), anti-Gr-1 (FITC, RB6-8C5) and anti-F4/80 (APC-F4/80), all from BioLegend. Samples were run on an LSR Fortessa (BD) or NovoCyte (ACEA). Cell sorting was performed by Beckman Moflo Astrios Eq. Data were analyzed using FlowJo v10 software

ROS and mitochondrial ROS measurements

Total ROS was measured by Reactive Oxygen Species Assay Kit from Beyotime (Cat. S0033), cells were incubated with antibody after stimulation in the incubator for 30 min, then rinsed in PBS and analyzed by NovoCyte flow cytometers. Mitochondrial ROS was measured by incubation with 5mM MitoSOX in PBS for 30 min at 37 °C. The cells were rinsed in cold PBS and analyzed by NovoCyte flow cytometers. Data were processed using FlowJo v10 software.

Western blotting and immunoprecipitation

Cells were lysed in cell lysis buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.05% bromophenol blue, 1 mM PMSF, and protease inhibitors), the samples were then boiled and separated on 10% or 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% BSA in TBST (0.5 M NaCl, Tris-HCl, pH 7.5, and 0.05% [vol/vol] Tween 20) for 1h at room temperature. After incubation with primary and secondary antibodies, ECL blotting reagents (Thermo Fisher) were used for immunoblot detection. For transfection and co-Immunoprecipitation, constructs were transfected into HEK293T cells using polyethyleneimine (PEI) or Lipofectamine 2000. After 24 h, the cells were collected and resuspended in cell lysis buffer, cell lysates were immunoprecipitated by incubation of lysates with 1–10 μg antibody overnight at 4°C and pulldown of antibody–protein precipitates with SureBeads Protein A (or protein G) magnetic beads (Bio-Rad Laboratories). The presence of immunocomplex proteins was determined by Western blot analysis.

ASC Oligomerization and ASC Speck Formation

ASC oligomerization and speck formation conditions were as previously described (15). After stimulation, BMDMs were rinsed in PBS and 500 µL ice-cold buffer (50 mM Tris-HCl pH 7.6, 0.5% Triton X-100, 0.1 mM PMSF, and a protease inhibitor cocktail) was added. Cells were scraped, lysed, and centrifuged at 330 x g for 10 min and the pellets were washed twice and re-suspended in 500 µL PBS. Disuccinimidyl suberate (2 mM) was added to the resuspended pellets and then incubated at room temperature for 30 min with rotation. Samples were then centrifuged and re-suspended in SDS loading buffer for western blotting.

For ASC speck formation, BMDMs were seeded and cultured overnight on glass coverslips. The following day, the cells were primed with LPS and treated with ATP in the presence or absence of the indicated inhibitors. The cells were fixed in 4% paraformaldehyde followed by ASC and DAPI staining.

Immunofluorescence Staining and Confocal Microscopy

Briefly, 2×10^5 BMBMs were plated on coverslips overnight. After blocking with 5% BSA (Sigma), cells were incubated with primary antibodies overnight at 4℃ in PBS containing 1% BSA. The following day, after three washes in PBS with Tween 20 (PBST), the cells were incubated with secondary antibodies in PBS for 1h at room temperature and rinsed in PBST, then mounted with mounting media containing DAPI (Sigma). Confocal microscopy analyses were carried out using a Zeiss LSM880/800 or Nikon A1R.

ELISA

Supernatants from cell cultures and sera were collected and the concentrations of IL-1β, IL-18, IL-6 and TNF-α (all from Thermo Fisher) were determined according to the manufacturer's instructions.

Quantitative PCR (qPCR) with reverse transcription

RNA was extracted using a total RNA extraction reagent (Vazyme Biotech). Complementary DNA was synthesized using HiScript® II Reverse Transcriptase (Vazyme Biotech) according to the manufacturer's instructions. qPCR was performed

using SYBR Green (Vazyme Biotech) on a CFX96 Touch Real-Time PCR (BioRad). All samples were individually normalized to β-Actin. The primers were displayed in table 1.

MS analysis

MINK1 and NLRP3 were co-transfected into HEK293T cells using PEI. After 24h, cells were collected and resuspended in cold cell lysis buffer. Extracts were immunoprecipitated with Flag-M2 monoclonal antibody–agarose beads and then dissolved in sample buffer. Cells lysates were separated on SDS-PADE and followed by in-gel digestion, desalted and then analyzed with the assistance of the Tsinghua University Protein Research Technology Center. Data analysis was carried out with Maxquant (v1.6.8.0).

***In vivo* animal model**

Mink1$^{-/-}$ and WT mice were injected intraperitoneally with LPS or aluminum salts. For the sepsis model, mice were sacrificed 4 h after LPS challenge (25 mg/kg body weight), and the serum levels of IL-1β, IL-18, and TNF-α were measured by ELISA (Thermo Fisher). For the acute peritonitis model, mice were sacrificed 6 h after Alum (2 mg per mice) challenge, Flow cytometry was used to detect the proportion of neutrophils in the peritoneal flushing fluid, and the release of IL-1β and TNF-α were also detected by ELISA. For the survival model, mice were challenged with LPS (20 mg/kg body weight) and observed for up to 30h.

***In vitro* kinase activity assay**

The MINK1 kinase was immunoprecipitated in cell lysates of LPS stimulating macrophages and MEFs by MINK1 antibody and protein A/G-Sepharose. Beads were washed 3 times with lysis buffer and 2 times with 1 × kinase assay buffer. For kinase reaction in vitro, beads containing MINK1 were incubated with 20ug purified substrates Myelin Basic Protein (MBP), 50 μM ATP, 10 × kinase assay buffer and 1 × protease inhibitor cocktail, at 30°C for 30 min with flicking. All kinase assay reactions were stopped with 5 × SDS loading buffer and boiled at 100°C for 5 min, and then subjected to immunoblot analysis, IB with anti-thiophosphate antibody.

Human NLRP3-NEK7 symmetric docking

The input model is Cryo-EM structure of NLRP3 bound to NEK7 [Protein Data Bank (PDB) ID:6npy] (54, 55). A total of 100 models were relaxed by the relax application (56) within the Rosetta suite. The model with the lowest energy was used for symmetric docking. Point mutation S728A was introduced by the backrub application (57) in Rosetta. The models of mutants were again relaxed by Rosetta before docking.

Symmetric docking application (58) was used to dock. A total of 10,000 models were generated for each docking trial. After docking, the top 10 models with the lowest score were chosen as the candidates. The model with the lowest score among the largest cluster of the top 10 models was used as the representative model.

Statistical Analysis

All results are presented as the mean ± SEM or SD. All the data were tested for normal distributions. Statistical analysis was carried out using Student's t-test (two-tailed unpaired) for two groups, ANOVA for multi-group comparison, and the Kaplan-Meier method for mouse survival as indicated, all using GraphPad Prism 8 unless otherwise noted. Differences were considered significant when $p < 0.05$.

Acknowledgments: The authors thank Xinhui Song, Chun Guo, Jiajia Wang and Yingying Huang from the core facilities (Zhejiang University School of Medicine) for technical assistance in histology and FACS analysis. We are grateful to Prof. Fan Yang and Wenxuan Zhen for human NLRP3-NEK7 symmetric docking. We would like to thank the support from the Key Laboratory of Immunity and Inflammatory Diseases of Zhejiang Province. This work was supported by grants from the National Natural Science Foundation of China (31930038, 31770954 and 31530019 to L.L.).

Author contributions: K.Z., X.J., D.W., X.L., and L.L. designed the research; K.Z., X.J., C.Z., S.C., and S.W. performed the research; K.Z., X.J. and L.L. analyzed the data; K.Z., X.J., and L.L. wrote the paper; R.S., H.H., D.T., edited the paper.

Conflict of interest: Authors declare no competing interests.

References

1. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu Rev Immunol. 2011;29:707-35.
2. Evavold CL, Kagan JC. Inflammasomes: Threat-Assessment Organelles of the Innate Immune System. Immunity. 2019;51(4):609-24.
3. He Y, Hara H, Nunez G. Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends Biochem Sci. 2016;41(12):1012-21.
4. Broz P. Inflammasomes in Host Defense and Autoimmunity. Chimia (Aarau). 2016;70(12):853-5.
5. Broz P. Inflammasomes: Intracellular detection of extracellular bacteria. Cell Res. 2016;26(8):859-60.
6. Baroja-Mazo A, Martin-Sanchez F, Gomez AI, Martinez CM, Amores-Iniesta J, Compan V, Barbera-Cremades M, Yague J, Ruiz-Ortiz E, Anton J, Bujan S, Couillin I, Brough D, Arostegui JI, Pelegrin P. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. Nat Immunol. 2014;15(8):738-48.
7. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 2006;440(7081):237-41.
8. Schroder K, Zhou R, Tschopp J. The NLRP3 inflammasome: a sensor for metabolic danger? Science. 2010;327(5963):296-300.
9. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nat Immunol. 2011;12(5):408-15.
10. Kummer JA, Broekhuizen R, Everett H, Agostini L, Kuijk L, Martinon F, van Bruggen R, Tschopp J. Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. J Histochem Cytochem. 2007;55(5):443-52.
11. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol. 2016;16(7):407-20.
12. Carta S, Tassi S, Pettinati I, Delfino L, Dinarello CA, Rubartelli A. The rate of interleukin-1beta secretion in different myeloid cells varies with the extent of redox response to Toll-like receptor triggering. J Biol Chem. 2011;286(31):27069-80.
13. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol. 2019;19(8):477-89.
14. Song N, Liu ZS, Xue W, Bai ZF, Wang QY, Dai J, Liu X, Huang YJ, Cai H, Zhan XY, Han QY, Wang H, Chen Y, Li HY, Li AL, Zhang XM, Zhou T, Li T. NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation. Mol Cell. 2017;68(1):185-97 e6.
15. Guo C, Xie S, Chi Z, Zhang J, Liu Y, Zhang L, Zheng M, Zhang X, Xia D, Ke Y, Lu L, Wang D. Bile Acids Control Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome. Immunity. 2016;45(4):802-16.
16. Chi Z, Chen S, Xu T, Zhen W, Yu W, Jiang D, Guo X, Wang Z, Zhang K, Li M, Zhang J, Fang H, Yang D, Ye Q, Yang X, Lin H, Yang F, Zhang X, Wang D. Histone Deacetylase 3 Couples Mitochondria to Drive IL-1beta-Dependent Inflammation by Configuring Fatty Acid Oxidation.

611 Mol Cell. 2020;80(1):43-58 e7.
612 17. Stutz A, Kolbe CC, Stahl R, Horvath GL, Franklin BS, van Ray O, Brinkschulte R, Geyer M,
613 Meissner F, Latz E. NLRP3 inflammasome assembly is regulated by phosphorylation of the pyrin
614 domain. J Exp Med. 2017;214(6):1725-36.
615 18. Spalinger MR, Kasper S, Gottier C, Lang S, Atrott K, Vavricka SR, Scharl S, Raselli T, Frey-
616 Wagner I, Gutte PM, Grutter MG, Beer HD, Contassot E, Chan AC, Dai X, Rawlings DJ, Mair F, Becher
617 B, Falk W, Fried M, Rogler G, Scharl M. NLRP3 tyrosine phosphorylation is controlled by protein
618 tyrosine phosphatase PTPN22. J Clin Invest. 2016;126(11):4388.
619 19. Huang Y, Wang H, Hao Y, Lin H, Dong M, Ye J, Song L, Wang Y, Li Q, Shan B, Jiang Y, Li H,
620 Shao Z, Kroemer G, Zhang H, Bai L, Jin T, Wang C, Ma Y, Cai Y, Ding C, Liu S, Pan Y, Jiang W, Zhou
621 R. Myeloid PTEN promotes chemotherapy-induced NLRP3-inflammasome activation and
622 antitumour immunity. Nat Cell Biol. 2020;22(6):716-27.
623 20. Dan I, Watanabe NM, Kobayashi T, Yamashita-Suzuki K, Fukagaya Y, Kajikawa E, Kimura WK,
624 Nakashima TM, Matsumoto K, Ninomiya-Tsuji J, Kusumi A. Molecular cloning of MINK, a novel
625 member of mammalian GCK family kinases, which is up-regulated during postnatal mouse
626 cerebral development. FEBS Lett. 2000;469(1):19-23.
627 21. Dan I, Watanabe NM, Kusumi A. The Ste20 group kinases as regulators of MAP kinase
628 cascades. Trends Cell Biol. 2001;11(5):220-30.
629 22. Leong SY, Ong BK, Chu JJ. The role of Misshapen NCK-related kinase (MINK), a novel Ste20
630 family kinase, in the IRES-mediated protein translation of human enterovirus 71. PLoS Pathog.
631 2015;11(3):e1004686.
632 23. Nicke B, Bastien J, Khanna SJ, Warne PH, Cowling V, Cook SJ, Peters G, Delpuech O, Schulze
633 A, Berns K, Mullenders J, Beijersbergen RL, Bernards R, Ganesan TS, Downward J, Hancock DC.
634 Involvement of MINK, a Ste20 family kinase, in Ras oncogene-induced growth arrest in human
635 ovarian surface epithelial cells. Mol Cell. 2005;20(5):673-85.
636 24. Larhammar M, Huntwork-Rodriguez S, Rudhard Y, Sengupta-Ghosh A, Lewcock JW. The
637 Ste20 Family Kinases MAP4K4, MINK1, and TNIK Converge to Regulate Stress-Induced JNK
638 Signaling in Neurons. J Neurosci. 2017;37(46):11074-84.
639 25. Hussain NK, Hsin H, Huganir RL, Sheng M. MINK and TNIK differentially act on Rap2-
640 mediated signal transduction to regulate neuronal structure and AMPA receptor function. J
641 Neurosci. 2010;30(44):14786-94.
642 26. McCarty N, Paust S, Ikizawa K, Dan I, Li X, Cantor H. Signaling by the kinase MINK is essential
643 in the negative selection of autoreactive thymocytes. Nat Immunol. 2005;6(1):65-72.
644 27. Yue M, Luo D, Yu S, Liu P, Zhou Q, Hu M, Liu Y, Wang S, Huang Q, Niu Y, Lu L, Hu H.
645 Misshapen/NIK-related kinase (MINK1) is involved in platelet function, hemostasis, and thrombus
646 formation. Blood. 2016;127(7):927-37.
647 28. Meng Z, Moroishi T, Mottier-Pavie V, Plouffe SW, Hansen CG, Hong AW, Park HW, Mo JS, Lu
648 W, Lu S, Flores F, Yu FX, Halder G, Guan KL. MAP4K family kinases act in parallel to MST1/2 to
649 activate LATS1/2 in the Hippo pathway. Nat Commun. 2015;6:8357.
650 29. Moroishi T, Hayashi T, Pan WW, Fujita Y, Holt MV, Qin J, Carson DA, Guan KL. The Hippo
651 Pathway Kinases LATS1/2 Suppress Cancer Immunity. Cell. 2016;167(6):1525-39 e17.
652 30. Fu G, Xu Q, Qiu Y, Jin X, Xu T, Dong S, Wang J, Ke Y, Hu H, Cao X, Wang D, Cantor H, Gao X,
653 Lu L. Suppression of Th17 cell differentiation by misshapen/NIK-related kinase MINK1. J Exp Med.
654 2017;214(5):1453-69.

31. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol. 2009;183(2):787-91.
32. Sutterwala FS, Ogura Y, Zamboni DS, Roy CR, Flavell RA. NALP3: a key player in caspase-1 activation. J Endotoxin Res. 2006;12(4):251-6.
33. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol. 2008;9(8):847-56.
34. Hyodo T, Ito S, Hasegawa H, Asano E, Maeda M, Urano T, Takahashi M, Hamaguchi M, Senga T. Misshapen-like kinase 1 (MINK1) is a novel component of striatin-interacting phosphatase and kinase (STRIPAK) and is required for the completion of cytokinesis. J Biol Chem. 2012;287(30):25019-29.
35. Kaneko S, Chen X, Lu P, Yao X, Wright TG, Rajurkar M, Kariya K, Mao J, Ip YT, Xu L. Smad inhibition by the Ste20 kinase Misshapen. Proc Natl Acad Sci U S A. 2011;108(27):11127-32.
36. Liu X, Pichulik T, Wolz OO, Dang TM, Stutz A, Dillen C, Delmiro Garcia M, Kraus H, Dickhofer S, Daiber E, Munzenmayer L, Wahl S, Rieber N, Kummerle-Deschner J, Yazdi A, Franz-Wachtel M, Macek B, Radsak M, Vogel S, Schulte B, Walz JS, Hartl D, Latz E, Stilgenbauer S, Grimbacher B, Miller L, Brunner C, Wolz C, Weber ANR. Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase. J Allergy Clin Immunol. 2017;140(4):1054-67 e10.
37. Martinez GJ. MINK1: The missing link between ROS and its inhibition of Th17 cells. J Exp Med. 2017;214(5):1205-6.
38. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature. 2011;469(7329):221-5.
39. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol. 2010;10(3):210-5.
40. Du K, Ramachandran A, Weemhoff JL, Woolbright BL, Jaeschke AH, Chao X, Ding WX, Jaeschke H. Mito-tempo protects against acute liver injury but induces limited secondary apoptosis during the late phase of acetaminophen hepatotoxicity. Arch Toxicol. 2019;93(1):163-78.
41. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, Sadler JJ, Knepper-Adrian V, Han R, Qiao L, Eisenbarth SC, Nauseef WM, Cassel SL, Sutterwala FS. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. Immunity. 2013;39(2):311-23.
42. Dan Dunn J, Alvarez LA, Zhang X, Soldati T. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. Redox Biol. 2015;6:472-85.
43. Liu T, Wang L, Liang P, Wang X, Liu Y, Cai J, She Y, Wang D, Wang Z, Guo Z, Bates S, Xia X, Huang J, Cui J. USP19 suppresses inflammation and promotes M2-like macrophage polarization by manipulating NLRP3 function via autophagy. Cell Mol Immunol. 2020.
44. Bauernfeind F, Bartok E, Rieger A, Franchi L, Nunez G, Hornung V. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. J Immunol. 2011;187(2):613-7.
45. Tassi S, Carta S, Delfino L, Caorsi R, Martini A, Gattorno M, Rubartelli A. Altered redox state of monocytes from cryopyrin-associated periodic syndromes causes accelerated IL-1beta

699 secretion. Proc Natl Acad Sci U S A. 2010;107(21):9789-94.
700 46. Sanchez-Rodriguez R, Munari F, Angioni R, Venegas F, Agnellini A, Castro-Gil MP, Castegna
701 A, Luisetto R, Viola A, Canton M. Targeting monoamine oxidase to dampen NLRP3 inflammasome
702 activation in inflammation. Cell Mol Immunol. 2020.
703 47. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links
704 oxidative stress to inflammasome activation. Nat Immunol. 2010;11(2):136-40.
705 48. Park YJ, Yoon SJ, Suh HW, Kim DO, Park JR, Jung H, Kim TD, Yoon SR, Min JK, Na HJ, Lee SJ,
706 Lee HG, Lee YH, Lee HB, Choi I. TXNIP deficiency exacerbates endotoxic shock via the induction of
707 excessive nitric oxide synthesis. PLoS Pathog. 2013;9(10):e1003646.
708 49. Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, Becker C, Franchi L,
709 Yoshihara E, Chen Z, Mullooly N, Mielke LA, Harris J, Coll RC, Mills KH, Mok KH, Newsholme P,
710 Nunez G, Yodoi J, Kahn SE, Lavelle EC, O'Neill LA. Activation of the NLRP3 inflammasome by islet
711 amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. Nat
712 Immunol. 2010;11(10):897-904.
713 50. Zhao C, Gillette DD, Li X, Zhang Z, Wen H. Nuclear factor E2-related factor-2 (Nrf2) is required
714 for NLRP3 and AIM2 inflammasome activation. J Biol Chem. 2014;289(24):17020-9.
715 51. Wegiel B, Larsen R, Gallo D, Chin BY, Harris C, Mannam P, Kaczmarek E, Lee PJ, Zuckerbraun
716 BS, Flavell R, Soares MP, Otterbein LE. Macrophages sense and kill bacteria through carbon
717 monoxide-dependent inflammasome activation. J Clin Invest. 2014;124(11):4926-40.
718 52. Jhang JJ, Yen GC. The role of Nrf2 in NLRP3 inflammasome activation. Cell Mol Immunol.
719 2017;14(12):1011-2.
720 53. Freeman TL, Swartz TH. Targeting the NLRP3 Inflammasome in Severe COVID-19. Front
721 Immunol. 2020;11:1518.
722 54. Sharif H, Wang L, Wang WL, Magupalli VG, Andreeva L, Qiao Q, Hauenstein AV, Wu Z, Nunez
723 G, Mao Y, Wu H. Structural mechanism for NEK7-licensed activation of NLRP3 inflammasome.
724 Nature. 2019;570(7761):338-43.
725 55. Hu Z, Yan C, Liu P, Huang Z, Ma R, Zhang C, Wang R, Zhang Y, Martinon F, Miao D, Deng H,
726 Wang J, Chang J, Chai J. Crystal structure of NLRC4 reveals its autoinhibition mechanism. Science.
727 2013;341(6142):172-5.
728 56. Conway P, Tyka MD, DiMaio F, Konerding DE, Baker D. Relaxation of backbone bond
729 geometry improves protein energy landscape modeling. Protein Sci. 2014;23(1):47-55.
730 57. Davis IW, Arendall WB, 3rd, Richardson DC, Richardson JS. The backrub motion: how protein
731 backbone shrugs when a sidechain dances. Structure. 2006;14(2):265-74.
732 58. Andre I, Bradley P, Wang C, Baker D. Prediction of the structure of symmetrical protein
733 assemblies. Proc Natl Acad Sci U S A. 2007;104(45):17656-61.
734
735

736 **Figure legends**

737

738 **Fig. 1. Myeloid MINK1 is essential for NLRP3 inflammasome activation. (A and**
739 **B)** BMDMs from *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice primed with LPS and stimulated with the
740 different secondary signals including ATP, nigericin, aluminum salts (Alum) and
741 monosodium urate crystals (MSU). Supernatants (SN) and cell extracts (Lysate) were
742 analyzed by immunoblotting (A). Supernatants IL-1β, IL-18 were analyzed ELISA (B).
743 **(C and D)** BMDMs from *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice primed with LPS and stimulated
744 with ATP, poly (dA:dT) and Salmonella. SN and Lysate were analyzed by
745 immunoblotting (C). SN IL-1β was also analyzed by ELISA (D). **(E and F)** BMDMs
746 from *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice primed with LPS and stimulated with ATP, followed
747 by immunoblotting for ASC oligomerization analysis in cross-linked cytosolic pellets
748 (E); representative immunofluorescence images and quantification of ASC speck
749 formation are shown in (F). *$p < 0.05$, two-tailed unpaired student's t-test was used for
750 Fig. B and D. The ELISA and western blot results are representative of three
751 independent experiments.

752

753 **Fig. 2. MINK1 ablation ameliorates acute inflammation. (A)** *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$
754 mice were intraperitoneally injected with LPS (25 mg/kg) for 4 h, IL-1β, IL-18, IL-6
755 and TNF-α in serum were measured by ELISA. Data are the mean ± SD (n=20
756 mice/group). **(B)** The survival rate of *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice injected with LPS (20
757 mg/kg, n=14 in *Mink1*$^{+/+}$ and n=16 in *Mink1*$^{-/-}$). **(C and D)** Surface staining of F4/80
758 and FLICA on macrophages from peritoneal cavity, spleen and blood from *Mink1*$^{+/+}$
759 and *Mink1*$^{-/-}$ mice to determine the proportion of activated caspase-1 macrophages (C)
760 and the intensity of caspase-1 activation (D). Numbers in or adjacent to outlined areas
761 (or in quadrants) indicate the percentages of cells in each throughout. **(E)**
762 Representative histology of liver and lung (hematoxylin and eosin) from *Mink1*$^{-/-}$ and
763 WT mice after LPS (25 mg/kg) injection for 4h. Scale bar represents 100 μm. **(F and**
764 **G)** *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice were intraperitoneally injected with alum (2 mg per

765 mice) for 6h, peritoneal fluid was washout for cell surface staining to determine the
766 percentages and cell number of neutrophils between two groups (F) Data are the mean
767 ± SD (n=6 mice/group). Peritoneal IL-1β and TNF-α from *Mink1*$^{-/-}$ and WT mice were
768 also detected by ELISA (G). *p < 0.05, *p < 0.01, ***p <0.001, two-tailed unpaired
769 student's t-test for Fig. A, F and G, and Kaplan-Meier method for mouse survival (B).
770 The ELISA and Flow cytometry results are representative of three independent
771 experiments.
772
773 **Fig. 3. MINK1 directly interacts with NLRP3 and regulates NLRP3 activation**
774 **through its kinase activity. (A)** Schematic diagram of the structure of MINK1 protein.
775 **(B, C and D)** BMDMs from *Mink1*$^{-/-}$ mice primed with LPS retrovirally transduced
776 with pHAGE empty vector, pHAGE-CNH domain, or pHAGE-Kinase domain
777 stimulated with ATP. Supernatants (SN) and cell extracts (Lysate) were examined by
778 immunoblotting (B). IL-1β and TNF-α secretion was determined by ELISA (C and D).
779 **(E and F)** Reconstruction of NLRP3 activation in HEK293T cells by overexpressing
780 NLRP3 inflammasome activation related components with HA-MINK1 or HA-
781 MINK1-K54R. Supernatants (SN) and cell extracts (Lysate) were determined by
782 Immunoblot (E). IL-1β secretion was determined by ELISA (F). **(G)** IP and immunoblot
783 analysis of the interaction between endogenous MINK1 and NLRP3 inflammasome
784 activation related components in BMDMs primed with LPS and then stimulated with
785 ATP. **(H)** Representative confocal images of HEK293T overexpressed with HA-
786 MINK1 (Red) and Flag-NLRP3 (Green). Scale bar represents 50 μm**. (I)** IP and
787 immunoblot analysis of the interaction between Flag-MINK1 and HA-NLRP3. **(J)** IP
788 and immunoblot analysis of the interaction between Flag-NLRP3 and HA-MINK1 or
789 HA-MINK1-K54R. **(K)** IP and immunoblot analysis of the interaction between HA-
790 NLRP3 and Flag-Kinase domain. **(L)** IP and immunoblot analysis of the interaction
791 between HA-MINK1 and full-length or truncated NLRP3. *p < 0.05, one-way ANOVA
792 was used for Fig. C and D, two-tailed unpaired student's t-test was used for Fig. F. The
793 ELISA, IF and western blot results are representative of three independent experiments.

Fig. 4. MINK1 mediated NLRP3 phosphorylation at Ser725 is critical for inflammasome activation. (A) Phos-tag™ SDS-PAGE was used for detection of phosphorylated NLRP3 in BMDMs primed with LPS and stimulated with nigericin. **(B)** IP was performed after overexpressing MINK1 and NLRP3 in HEK293T cell, followed by phosphorylation mass spectrometry to show the phosphorylated residue. **(C and D)** Reconstruction of NLRP3 activation in HEK293T cells, supernatants (SN) and cell extracts (Lysate) were determined by Immunoblot (C). The IL-1β release was determined by ELISA (D). **(E and F)** ELISA analysis of IL-1β, IL-18, and TNF-α in culture supernatants (E) or immunoblot analysis of p17 and p20 (F) from *Nlrp3*$^{-/-}$ BMDMs transduced with mouse WT or mutant NLRP3 expressing lentivirus, and then primed with LPS and stimulated with ATP. **(G)** IP and immunoblot analysis of the aggregation of NLRP3 after overexpressing HA-NLRP3 with different Flag-NLRP3 in HEK293T. **(H)** IP and immunoblot analysis of the aggregation of NLRP3 after overexpressing Flag-MINK1, Flag-MINK1-K54R and different tag-NLRP3 in HEK293T. **(I, J and K)** The structure model of WT NLRP3-NEK is shown in blue, Serine 728 is shown in red. The inset shows the detailed confirmation (J) and the S728A mutant is shown in (K). The inset shows the detailed conformation. The score (in the unit of R.E.U.) reflecting the structural stability was calculated by Rosetta for WT NLRP3-NEK and S728A mutant (I). *p <0.05, **p <0.01, ***p <0.001, one-way ANOVA was used for Fig. D and E, two-tailed unpaired Student's t-test was used for Fig I. The ELISA and western blot results are representative of three independent experiments.

Fig. 5. ROS activate MINK1 kinase to prime the activation of NLRP3. (A) LPS primed BMDMs from *Mink1*$^{-/-}$ and WT mice were stimulated with ATP, total ROS and mitochondrial ROS levels were measured by ROS and MitoSOX incorporation using flow cytometry. **(B and C)** LPS primed BMDMs from *Mink1*$^{-/-}$ and WT mice were treated with NAC (3 mM) 30 min before imiquimod (a small-molecule ligand of Toll-

like receptor-7 (TLR7)) stimulated. Supernatants (SN) and cell extracts (Lysate) were analyzed by immunoblot (C), and supernatants were also analyzed by ELISA for IL-1β (B). **(D, E, and F)** LPS primed BMDMs were treated with NAC 30 min before imiquimod stimulation, MINK1 was immunoprecipitated, and in vitro kinase assays were performed with myelin basic protein as the substrate in the presence of ATP-γ-S. The reaction products were immunoblotted with anti-thiophosphate ester antibody (D). Supernatants were analyzed by ELISA for IL-1β (E), and fold change of kinase activity was quantified and presented in (F). *p <0.05, **p <0.01, ***p <0.001, two-tailed unpaired Student's t-test was used for Fig. F and ANOVA was used for Fig. B and E. The Flow cytometry, ELISA and western blot results are representative of three independent experiments.

Fig. 6. NAC significantly alleviates the acute inflammatory response mediated by NLRP3 activation in both human and mouse cells. (A) Schematic diagram of NAC treatment model. **(B and C)** 8-week mice were intraperitoneally injected with LPS (25 mg/kg), followed by different NAC treatments at different time points. Serum was collected to detect IL-1β, IL-18. Data are the mean ± SD (n=6 mice/group). **(D)** Surface staining of F4/80 and FLICA on macrophages in spleen from C57BL/6 (B6) mice to determine the intensity of caspase-1 activation. **(E)** Surface staining of F4/80 and FLICA on peritoneal macrophages from *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice to determine the proportion of activated caspase-1 macrophages. **(F)** Survival rate of *Mink1*$^{+/+}$ and *Mink1*$^{-/-}$ mice injected with 20 mg/kg LPS. **(G and H)** PBMCs were freshly isolated from healthy donors, LPS primed PBMCs with NAC or Mito-TEMPO before imiquimod or nigericin stimulated, supernatant IL-1β was analyzed by ELISA. **(I)** LPS-primed THP-1 cells treated with Mito-TEMPO before nigericin stimulated. Supernatants (SN) and cell extracts (Lysate) were analyzed by immunoblot. *p <0.05, **p <0.01, ***p <0.001, one way ANOVA was used for Fig. B, C, D, E, G and H, and Kaplan-Meier method was used for mouse survival Fig. F. The ELISA and western blot results are representative of three independent experiments.