NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component

Hexin Shi1, Ying Wang1, Xiaohong Li1, Xiaoming Zhan1, Miao Tang1, Maggy Fina1, Lijing Su1, David Pratt1, Chun Hui Bu1, Sara Hildebrand1, Stephen Lyon1, Lindsay Scott1, Jiexia Quan1, Qihua Sun1, Jamie Russell1, Stephanie Arnett1, Peter Jurek1, Ding Chen2, Vladimir V Kravchenko3, John C Mathison3, Eva Marie Y Moresco1, Nancy I. Monson2, Richard J Ulevitch3 & Bruce Beutler1

The NLRP3 inflammasome responds to microbes and danger signals by processing and activating proinflammatory cytokines, including interleukin 1β (IL-1β) and IL-18. We found here that activation of the NLRP3 inflammasome was restricted to interphase of the cell cycle by NEK7, a serine-threonine kinase previously linked to mitosis. Activation of the NLRP3 inflammasome required NEK7, which bound to the leucine-rich repeat domain of NLRP3 in a kinase-independent manner downstream of the induction of mitochondrial reactive oxygen species (ROS). This interaction was necessary for the formation of a complex containing NLRP3 and the adaptor ASC, oligomerization of ASC and activation of caspase-1. NEK7 promoted the NLRP3-dependent cellular inflammatory response to intraperitoneal challenge with monosodium urate and the development of experimental autoimmune encephalitis in mice. Our findings suggest that NEK7 serves as a cellular switch that enforces mutual exclusivity of the inflammasome response and cell division.

Inflammasomes are multiprotein complexes that serve as platforms for the activation of caspase-1, which leads to the processing and secretion of interleukin 1β (IL-1β) and IL-18 and to the induction of pyroptosis, a form of programmed cell death. The NLRP3 inflammasome is activated in macrophages by a two-step process that involves priming through the activation of pathways that activate the transcription factor NF-kB before or simultaneously with exposure to a second NLRP3-specific trigger such as extracellular ATP, alum or the pore-forming toxin nigericin. Upon activation, NLRP3 and the adaptor ASC move from their positions at the endoplasmic reticulum and mitochondria, respectively, to form a complex at the perinuclear region, an event dependent on microtubule polymerization, acetylation of α-tubulin, and dynein-mediated transport of mitochondria from the periphery to the cell center. This complex recruits pro-caspase-1, which results in the activation of caspase-1.

Here, forward genetic analysis of inflammasome activation in C57BL/6J mice revealed NEK7, one of eleven NEK kinases present in vertebrates, as a component of the NLRP3 inflammasome in macrophages. NEK7 has also been linked to the formation of mitotic spindles and the separation of centromeres (with NEK6 and NEK9), to abscission during cytokinesis and to the regulation of interphase centrosomes. Our findings suggest that activation of the NLRP3 inflammasome and mitosis cannot occur simultaneously, in part because the quantity of NEK7 present in macrophages is sufficient for only one or the other. Thus, NEK7 acts as a switch between mitosis and competence for inflammasome activation, both of which require NEK7.

RESULTS

A Nek7 mutation impairs NLRP3 inflammasome activation

To identify regulators of NLRP3-mediated inflammation, we carried out a forward genetic screen in which we isolated macrophages from C57BL/6J mice carrying homozygous and heterozygous N-ethyl-N-nitrosourea–induced mutations and assessed the secretion of IL-1β from the cells in response to priming with lipopolysaccharide (LPS) followed by stimulation with nigericin (except where indicated otherwise, peritoneal macrophages were used throughout this study). We screened 16,816 G3 mice derived from 811 G1 grandsires bearing 49,590 non-synonymous mutations within the coding regions or splice junctions of 15,927 genes. We tested 16,328 mutations in 9,499 genes three or more times in the homozygous state; these included one or more putative null alleles of 924 genes. Among the phenovariants detected, several mice from a single pedigree displayed diminished IL-1β secretion by macrophages (Fig. 1a). We called this phenotype 'Cuties'.

To identify the Cuties (Cu) mutation, we sequenced coding exons and flanking splice junctions in genomic DNA from the G1 grandsire of the Cuties pedigree. We found 79 mutations with 97% coverage of target sequences at ten or more reads. We genotyped each of the 79 mutation sites in G3 mice of the Cuties pedigree and found that a mutation in Nek7 showed strongest linkage with the Cuties phenotype.
Nek7 mice, in contrast to Nek7+/ Cu/Cu mice, had normal macrophage responses to LPS-priming in the absence of further stimulation (Fig. 1b and (f)). This similar macrophage response between Nek7+Cu/Cu and Nek7−/− peritoneal macrophages, primed with LPS without further stimulation, was consistent with nonsense-mediated decay of the transcript (Fig. 1d). However, the concentration of NEK7 protein in Nek7−/− cells was on average 30% less than their Nek7+Cu/Cu counterparts (Fig. 1f). This decrease was significant with (red) or without (purple) the Bonferroni correction (P = 1.153 × 10−5). Horizontal lines indicate thresholds of P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 and **** P ≤ 0.0001 (unpaired, two-tailed Student’s t-test). Data are representative of one experiment (a,b), two experiments (c) or two independent experiments (d-f; mean and s.d. of n = 5 mice (Nek7+/Cu and Nek7−/− Cu/Cu) or 4 mice (Nek7−/− Cu/Cu and Nlrp3−/−) per genotype).

Further analysis demonstrated less production of IL-1β by LPS-primed Nek7+/Cu/Cu macrophages stimulated with ATP or alum than by their Nek7+/+ counterparts (Fig. 1d). We obtained similar results with bone marrow–derived macrophages and bone marrow–derived dendritic cells (Supplementary Fig. 1a,b). NLRP3-dependent production of IL-18 and pyroptosis were also impaired in LPS-primed Nek7+/Cu/Cu macrophages stimulated with nigericin, ATP or alum (Fig. 1c,f). The IL-1β and IL-18 responses of Nek7+/Cu/Cu and Nek7−/− Cu/Cu macrophages to infection with Escherichia coli or Citrobacter rodentium, mediated by non-canonical caspase-11-dependent NLRP3 signaling14, were defective (Fig. 1d,e); however, the programmed death response that is independent of NLRP3 but dependent on caspase-11 was normal (Fig. 1f). The production of IL-1β and IL-18 and release of lactate dehydrogenase (LDH) into the culture medium, presented relative to total lactate dehydrogenase activity in lysates of unstimulated cells. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 and **** P ≤ 0.0001 (unpaired, two-tailed Student’s t-test). Data are representative of one experiment (a,b), two experiments (c) or two independent experiments (d-f; mean and s.d. of n = 5 mice (Nek7+/Cu and Nek7−/− Cu/Cu) or 4 mice (Nek7−/− Cu/Cu and Nlrp3−/−) per genotype).

by a semidominant model of transmission (−log10 P value (non-linkage) = 4.938) (Fig. 1b). The mutation, a T-to-A transversion at chromosome 1, was predicted to cause substitution of a premature stop codon for the cysteine at position 53 of the 302-amino acid Nek7 protein. We detected no Nek7 protein in Nek7−/− Cu/Cu macrophages, consistent with nonsense-mediated decay of the transcript (Fig. 1c).

Nek7+/+ mice seemed to be normal and showed no internal anatomical abnormalities (data not shown). At birth, Nek7−/− Cu/Cu mice were similar in size to their littermates, but 2 months of age, they weighed on average 30% less than their Nek7+/+ or Nek7+/Cu/siblings (data not shown). Nek7−/− Cu/Cu mice had an abnormal gait and slight paresis of the limbs and were infertile (data not shown). Consistent with a published report11, crosses of heterozygous mutant mice (on the C57BL/6j background) revealed non-Mendelian transmission ratios of the mutation, with Nek7+/+, Nek7+/Cu and Nek7−/− Cu/Cu mice representing 34.9%, 60.4% and 4.7%, respectively, of the offspring at birth.

Figure 1 Impaired activation of the NLRP3 inflammasome in macrophages from Cuties mice. (a) Enzyme-linked immunosorbent assay (ELISA) of IL-1β in supernatants of wild-type peritoneal macrophages (WT) and Nek7+/Cu (REF), Nek7+/− Cu (HET) or Nek7+/− Cu/Cu (VAR) peritoneal macrophages from mice of the Cuties pedigree, primed with LPS and treated with nigericin. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±s.d.). (b) Analysis of the linkage of 79 mutations (symbols along plotted lines) identified in the G1 founder of the Cuties pedigree to the Cuties phenotype, presented as a Manhattan plot with −log10 P values plotted against the chromosomal positions of the mutations, identifying linkage of a mutation in Nek7 with the Cuties phenotype via a semidominant transmission model (P = 1.153 × 10−5). Horizontal lines indicate thresholds of P = 0.05 with (red) or without (purple) the Bonferroni correction. (c) Immunoblot analysis of Nek7 and α-tubulin (loading control throughout) in Nek7+/+, Nek7−/− Cu and Nek7+/− Cu/Cu peritoneal macrophages. (d,e) ELISA of (d) IL-1β and (e) IL-18 in supernatants of Nek7+/+, Nek7−/− Cu, Nek7+/− Cu/Cu and Nlrp3−/− peritoneal macrophages primed with LPS without further stimulation (None) or primed with LPS and treated with various inflammasome stimuli (horizontal axes). (f) Pyroptosis of peritoneal macrophages treated as in (d,e), assessed as release of lactate dehydrogenase (LDH) into the culture medium, presented relative to total lactate dehydrogenase activity in lysates of unstimulated cells. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 and **** P ≤ 0.0001 (unpaired, two-tailed Student’s t-test). Data are representative of one experiment (a,b), two experiments (c) or two independent experiments (d-f; mean and s.d. of n = 5 mice (Nek7+/Cu and Nek7−/− Cu/Cu) or 4 mice (Nek7−/− Cu/Cu and Nlrp3−/−) per genotype).
colony-forming unit assays showed normal differentiation and proliferation of Nek7<sup>Cu/Cu</sup> bone marrow hematopoietic stem cells and myeloid progenitor cells in response to treatment with macrophage colony-stimulating factor (Supplementary Fig. 2b). The recruitment of exudate cells, macrophages and neutrophils to the peritoneal cavity in response to intraperitoneal injection of bioactive IL-1β was also normal in Nek7<sup>Cu/Cu</sup> mice (Supplementary Fig. 2c). Although depletion of NEK7 via RNA-mediated interference has been reported to result in apoptosis<sup>8</sup>, we observed a similar frequency of apoptotic cells among Nek7<sup>+/+</sup> macrophages and Nek7<sup>Cu/Cu</sup> macrophages before or after priming with LPS (Supplementary Fig. 2d).

We knocked down endogenous NEK7 via small interfering RNA (siRNA) in the mouse macrophage cell line J774A.1 and observed much less secretion of IL-1β in response to stimulation with nigericin or ATP after priming with LPS, relative to that of Nek7-sufficient J774A.1 cells (Supplementary Fig. 3a). Similarly, in HEK293T human embryonic kidney cells reconstituted with functional mouse NLRP3 inflammasomes, siRNA-mediated knockdown of endogenous NEK7 inhibited the secretion of IL-1β in response to stimulation with nigericin or ATP (Supplementary Fig. 3b). A null allele of Nek7 generated by CRISPR (the genome-editing approach based on clustered regularly interspaced short palindromic repeats and the endonuclease Cas9) reproduced the Cuties phenotype in homozygous (Nek7<sup>−/−</sup>) mice (Supplementary Fig. 3c). Notably, in a cultured human monocyte cell line (THP-1) and in primary human monocytes, knockdown of endogenous NEK7 resulted in reduced secretion of IL-1β in response to priming with LPS plus stimulation with nigericin or ATP, relative to that of Nek7-sufficient cells (Supplementary Fig. 3d,e), whereas LPS-induced secretion of IL-6 was unaffected by knockdown of NEK7 (Supplementary Fig. 3f). These findings indicated that NEK7 was necessary for activation of the NLRP3 inflammasome in mouse macrophages and human monocytes.

Promotion of cellular inflammatory responses by NEK7 in vivo

In vivo, intraperitoneal injection of monosodium urate crystals (MSU) induces NLRP3-dependent recruitment of inflammatory cells to and IL-1β production in the peritoneal cavity. When challenged with MSU, Nek7<sup>Cu/Cu</sup> mice showed impaired recruitment of total cells, neutrophils and F4/80<sup>+</sup> monocytes-macrophages to the peritoneal cavity relative to that of Nek7<sup>+/+</sup> mice (Fig. 2a). Bone marrow chimeras generated by reconstitution of irradiated Nek7<sup>+/+</sup> mice with either Nek7<sup>+/+</sup> bone marrow or Nek7<sup>−/−</sup> bone marrow responded similarly to non-chimeric Nek7<sup>+/+</sup> mice and Nek7<sup>Cu/Cu</sup> mice, respectively (Fig. 2b). MSU-induced secretion of IL-1β, as detected in lavage fluid, was also significantly less in chimeras that received Nek7<sup>−/−</sup> bone marrow than in those that received Nek7<sup>+/+</sup> bone marrow (Fig. 2c). Thus, NEK7 functioned in the hematopoietic compartment to promote an NLRP3-dependent inflammatory response to intraperitoneal challenge with MSU.

We also investigated whether Nek7<sup>−/−</sup> mice were protected against IL-1β-driven inflammatory disease in a model of experimental autoimmune encephalitis (EAE)<sup>15,16</sup>. Immunization of wild-type mice with recombiant human myelin oligodendrocyte glycoprotein induced EAE, with paralysis and infiltration of the CNS by cells of the immune system (data not shown). Nek7<sup>−/−</sup> mice exhibited reduced disease severity relative to that of wild-type mice (Fig. 2d), as well as less recruitment of lymphocytes (CD4<sup>+</sup>, CD8<sup>+</sup>, TCRγδ<sup>+</sup> or CD19<sup>+</sup> B cells), monocytes-microglia, and natural killer cells to the spinal cord (Fig. 2e). Thus, NEK7 was necessary for the development of EAE.

![Figure 2](https://example.com/figure2.png)

**Figure 2** NEK7 promotes an inflammatory response in vivo. (a,b) Quantification of peritoneal exudate cells (PECs), neutrophils (Ly6G<sup>+</sup>Ly6C<sup>−</sup>) and monocytes-macrophages (F4/80<sup>+</sup>) in the peritoneum of Nek7<sup>+/+</sup>, Nek7<sup>Cu/Cu</sup> and Nek7<sup>Cu/Cu</sup> mice (a) or chimeras generated by reconstitution of irradiated Nek7<sup>+/+</sup> host mice with Nek7<sup>+/+</sup> or Nek7<sup>−/−</sup> donor bone marrow (key) (b), assessed by flow cytometry. 6 h after intraperitoneal injection of MSU. Each symbol represents an individual mouse; small horizontal lines indicate the mean. (c) ELISA of IL-1β in the peritoneal lavage fluid of Nek7<sup>+/+</sup> mice at 6 h after injection of PBS (germline; n = 4) or of chimeras as in b at 6 h after injection of MSU (n = 4 host mice per donor genotype). (d) Clinical score of wild-type (WT), Nek7<sup>+/+</sup> and Nlrp3<sup>−/−</sup> mice 0–21 d after immunization with recombinant human myelin oligodendrocyte glycoprotein (for induction of EAE) on day 0. (e) Quantification of various cell types of the immune system (horizontal axes) in the spinal cords of mice 21 d after immunization as in d (n = 4 per genotype), assessed by flow cytometry. Subsets were gated as natural killer (NK) cells (NK1.1<sup>+</sup>CD3ε<sup>−</sup>), monocytes-microglia (CD11b<sup>+</sup>Gr1<sup>−</sup>), neutrophils (CD11b<sup>+</sup>Gr1<sup>+</sup>) or lymphocytes (CD45<sup>+</sup>CD11b<sup>−</sup>). **P < 0.05, ***P < 0.01, ****P < 0.001 and ***P < 0.0001, relative to wild-type in d (unpaired, two-tailed Student’s t-test). Data are representative of two independent experiments (a–c; mean and s.d. in c) or one experiment (d,e; mean ± s.e.m. in d; mean and s.d. in e).
Inflammasome assembly and activation of caspase-1 require NEK7

Mitochondrial generation of reactive oxygen species (ROS), calcium influx, and a decrease in cellular cyclic AMP (cAMP) have been reported to activate the NLRP3 inflammasome,17 although their effects have been disputed18. We found that LPS-primed Nek7+/+ or Nek7−/− macrophages underwent induction of similar amounts of mitochondrial ROS after treatment with nigericin (Fig. 3a). The ATP-induced influx of calcium into the cytoplasm was also similar in LPS-primed Nek7+/+ macrophages and Nek7−/− macrophages, although intracellular calcium levels decreased more rapidly in Nek7−/− macrophages than in Nek7+/+ macrophages over a 30-minute period after the administration of ATP (Fig. 3b). A camp, which might bind directly to NLRP3 to inhibit inflammasome assembly,17 was more abundant in Nek7−/− macrophages than in Nek7+/+ macrophages after priming with LPS plus stimulation with nigericin, and there was a similar trend toward larger amounts of cAMP in LPS-primed Nek7−/− macrophages treated with ATP than in their Nek7+/+ counterparts (Fig. 3c). Thus, activation of the NLRP3 inflammasome might have been partially impaired in the Nek7−/− macrophages due to a defective cAMP response in which elevated concentrations of cAMP inhibited inflammasome assembly.

Upon activation of NLRP3, ASC oligomerizes and forms a complex with NLRP3 at the perinuclear region19-21. The expression of NLRP3, ASC, pro-caspase-1 and pro-IL-1β was similar in Nek7+/+, Nek7−/− and Nek7−/− macrophages primed with LPS (Fig. 4a). Nek7 expression was unaffected by LPS priming in Nek7+/+ or Nlrp3−/− macrophages (Fig. 4a). However, after priming with LPS and stimulation with either nigericin or ATP, Nek7+/+ and Nek7−/− macrophages had less association of NLRP3 with ASC than did their Nek7+/+ counterparts (Fig. 4b), and they demonstrated a failure of ASC oligomerization as well (Fig. 4c). The abundance of acetylated α-tubulin, a regulator of NLRP3-ASC binding2, was similar in Nek7+/+, Nek7−/− and Nek7−/− macrophages (Fig. 4d,e). Mutant macrophages also secreted reduced amounts of mature IL-1β and active caspase-1 (p10 subunit) relative to those secreted by Nek7+/+ macrophages (Fig. 4a). These findings indicated that NEK7 was dispensable for the induction of core inflammasome components but...
was necessary for subsequent formation of the NLRP3-ASC complex and activation of caspase-1.

**NEK7 binding to NLRP3 promotes inflammasome assembly**

In unstimulated cells, NLRP3 homo-oligomerizes to form inactive preassembled complexes, which undergo conformational changes to form active inflammasome complexes containing ASC upon stimulation22. Consistent with that, we found NLRP3 in complexes ranging from approximately 125 kilodaltons to 1,000 kilodaltons in size in the mouse macrophage cell line RAW264.7, which lacks ASC expression23, after priming of cells with LPS, as assessed by gel-filtration chromatography of cell extracts (Fig. 5a). Priming of the cells with LPS plus treatment with nigericin resulted in a shift of NLRP3 to fractions of greater molecular mass; the recruitment of NLRP3 to these fractions was blocked by siRNA-mediated knockdown of NEK7 (Fig. 5a), which suggested that NLRP3 bound directly to NEK7 to form a complex upon stimulation of the inflammasome. Indeed, when co-expressed in HEK293T cells, NEK7 immunoprecipitated together with NLRP3 but not with NLRC4 or AIM2 (Fig. 5b). The NEK7-NLRP3 interaction was mediated by an intact leucine-rich repeat (LRR) domain of NLRP3 (Fig. 5c,d). Deletion of the amino-terminal 20 or 30 amino acids of NEK7 did not affect its binding to NLRP3 (Fig. 5e); deletions from the central or carboxy-terminal portion of NEK7 abrogated protein expression and these mutant NEK7 proteins could not be tested. We confirmed direct interaction via maltose-binding protein (MBP)–mediated precipitation of purified recombinant MBP-NEK7 and NLRP3 (Supplementary Fig. 4a). ASC did not interact directly

![Figure 5](image-url)

**Figure 5** NEK7 directly interacts with the LRR domain of NLRP3 to promote inflammasome assembly. (a) Immunoblot analysis of lysates of RAW264.7 cells transfected with non-targeting control siRNA (NC) or Nek7-specific siRNA (siNek7), then primed with LPS and left unstimulated (top) or stimulated with nigericin (below), then fractionated by gel-filtration chromatography (fractions sizes at top). (b) Immuno precipitation and immunobl ot analysis of the interaction between hemagglutinin (HA)-tagged NEK7 and Flag-tagged components of the NLRP3 inflammasome, NLRC4, or AIM2 in HEK293T cells transfected to overexpress HA-tagged NEK7 (HA-Nek7) alone (–) or together with vector encoding Flag-tagged pro-IL-1β, ASC, pro-caspase-1, NLRP3, NLRC4 or AIM2 (above lanes) (b), vector encoding Flag-tagged full-length NLRP3 or truncations of NLRP3 containing only residues 1–90 (pyrin domain) (PYD), 91–710 (nucleotide-binding domain) (NBD) or 711–1033 (LRR domain) (LRR) (above lanes) (c) or vector encoding Flag-tagged full-length NLRP3 or truncations of NLRP3 with deletion (del) of various residues in the LRR domain (above lanes) (d), or transfected with vector encoding Flag-tagged NLRP3 and HA-tagged full-length NEK7 or mutants lacking (del) various residues (above lanes) (e). (f) Immunoassay of the endogenous NEK7-NLRP3 association in wild-type peritoneal macrophages primed with LPS and left unstimulated (–) or stimulated (+) with nigericin or ATP (above lanes), assessed by immunoprecipitation with immunoglobulin G (IgG), as a control, or with anti-NEK7, followed by immunoblot analysis with anti-NLRP3 or anti-NEK7. (g) Immunoblot analysis of lysates of wild-type peritoneal macrophages primed with LPS and left unstimulated (top) or stimulated with nigericin (below), fractionated by gel-filtration chromatography (fractions presented as in a). (h) Immunoprecipitation and immunoblot analyses (as in b) of lysates of HEK293T cells transfected to overexpress HA-tagged NEK7 alone (–) or together with vector expressing Flag-tagged human NLRP3 (Flag-hNLRP3), in the wild-type form (WT) or mutant NLRP3 with the substitution G755A or G755R (above lanes). (i) ELISA of IL-1β in supernatants of wild-type peritoneal macrophages (Nlrp3+/+) or mutant peritoneal macrophages homozygous for the mutation resulting in the D946G substitution (Nlrp3Δ946G/D946G), primed with LPS and stimulated with nigericin. Each symbol represents an individual mouse; small horizontal lines indicate the mean (s.d.). (j) ELISA and immunoblot analyses (as in b) of lysates of HEK293T cells transfected to overexpress HA-tagged NEK7 alone (–) or together with vector expressing Flag-tagged wild-type NLRP3 (WT) or mutant NLRP3 with the D946G substitution (above lanes). Data are representative of two independent experiments.
with MBP-NEK7 but was recruited to the NEK7-NLRP3 complex (Supplementary Fig. 4b).

In LPS-primed macrophages, the interaction between endogenous NEK7 and NLRP3 was substantially enhanced by stimulation with nigericin or ATP (Fig. 5f). Moreover, gel-filtration chromatography of extracts of macrophages primed with LPS and treated with nigericin demonstrated co-elution of NEK7, NLRP3 and ASC and a shift to fractions of greater molecular mass, results not observed for extracts of LPS-primed macrophages (Fig. 5g). The NEK7-NLRP3 interaction was partially dependent on microtubule polymerization (Supplementary Fig. 5a). However, cilobrevin D, an inhibitor of dynein that prevents formation of the NLRP3-ASC complex at the perinuclear region,4 had no effect on the NEK7-NLRP3 interaction (Supplementary Fig. 5a). The NEK7-NLRP3 interaction was also dependent on ROS-induced phosphorylation of NEK7. Treatment of J774A.1 cell lysates with the nonspecific phosphatase CIP (Supplementary Fig. 5b) or the ROS scavenger N-acetylcysteine (Supplementary Fig. 5c) diminished both the phosphorylation of NEK7 and the interaction between NEK7 and NLRP3. The production of IL-1β by J774A.1 cells in response to priming with LPS plus stimulation with ATP was diminished in a dose-dependent way by treatment with N-acetylcysteine (Supplementary Fig. 5d).

Mutant NLRP3 proteins containing the substitution G755A or G755R in the LRR domain, each of which is associated with neonatal onset multisystem inflammatory disease24–26, showed greater binding to NEK7 than did wild-type NLRP3 in HEK293T cells (Fig. 5h). Conversely, a hypomorphic NLRP3 missense mutant (D946G) that failed to support the secretion of IL-1β by LPS-primed macrophages treated with nigericin showed less binding to NEK7 than did wild-type NLRP3 (Fig. 5i,j). Together these data suggested that inflammasome assembly required the physical association of phosphorylated NEK7 with NLRP3.

**NEK7 kinase activity is dispensable for NLRP3 activation**

We reconstituted HEK293T cells with functional NLRP3 inflammasomes and showed that overexpression of either wild-type NEK7 or kinase-inactive NEK7 (with the K64M substitution)8 (Supplementary Fig. 4c) enhanced secretion of mature IL-1β into the culture medium (Fig. 6a). Wild-type NEK7 and NEK7(K64M) immunoprecipitated together with similar amounts of NLRP3 in lysates of HEK293T cells (Fig. 6b), and we also observed a direct interaction between recombinant MBP-NEK7(K64M) and NLRP3 (Supplementary Fig. 4a). Moreover, both NEK7 and NEK7(K64M) promoted recruitment of NLRP3 into protein complexes of large molecular mass without changing total NLRP3 expression substantially (Fig. 6c). Finally, when introduced by electroporation into Nek7Cu/Cu macrophages, both wild-type NEK7 and NEK7(K64M) restored the secretion of IL-1β in response to priming with LPS plus treatment with nigericin or ATP (Fig. 6d).

We found that Nek6, a related member of the NEK family that is 87% identical to NEK7 in its kinase domain27, failed to bind NLRP3 (Supplementary Fig. 6a). It was also not required for macrophage production of IL-1β in response to activators of NLRP3, NLRC4 or AIM2 inflammasomes (Supplementary Fig. 6b,c). These results indicated that the kinase activity of NEK7 was nonessential for activation of the NLRP3 inflammasome.

**Blockade of NLRP3 inflammasome activation in mitotic cells**

Since NEK7 is required for both activation of the NLRP3 inflammasome and mitosis, we investigated the interaction between NEK7 and NLRP3 during mitosis versus interphase of the cell cycle. Endogenous NEK7 and NLRP3 immunoprecipitated together from both mitotic and interphase J774.1 cells, with a greater amount of NLRP3 bound to NEK7 during interphase than during mitosis (Fig. 7a). In cells in interphase, priming with LPS plus stimulation with nigericin increased the amount of NLRP3 interacting with NEK7 relative to that in their unstimulated counterparts, but it did not have this effect on mitotic cells (Fig. 7a). Moreover, stimulated cells in interphase displayed more activation of caspase-1 than that of cells in mitosis (Fig. 7b). 25% of stimulated cells in interphase expressed activated caspase-1, compared with only 2% of stimulated mitotic cells (Fig. 7b). Consistent with those data, J774.1 cells synchronized by chemical arrest at the G2-M phase ‘border’28 and released to synchronize enter mitosis (Fig. 7c, left) exhibited diminished NLRP3-NEK7 interaction compared with that of their unsynchronized counterparts (Fig. 7c, right) and produced less IL-1β in response to treatment with LPS plus nigericin than did their unsynchronized counterparts.
Figure 7  Activation of the NLRP3 inflammasome is blocked during mitosis. (a) Immunoprecipitation and immunoblot analysis of the NEK7-NLRP3 association in LPS-primed J774A.1 mitotic (M) or interphase (I) cells, with (+) or without (−) overexpression of HA-NEK7 and treatment with nigericin (above lanes). (b) Flow cytometry analyzing activated caspase-1 in mitotic or interphase J774A.1 cells primed with LPS and left unstimulated or stimulated with nigericin, together with fluorescent FAM-FLICA—caspase-1 probe specific for activated caspase-1 (left), and the frequency of those cells among mitotic and interphase cells (right). (c) Flow cytometry (left) analyzing mitotic cells positive for histone H3 phosphorylated at Ser38 (p-H3(Ser38)) among J774A.1 cells without arrest, treated for 4 h with LPS (No RO-03306), or arrested at the G2-M phase 'border' by incubation for 20 h with RO-3306, with LPS added during the final 4 h, followed by no release (continuous) or release from arrest by incubation for 20 min, 1 h or 6 h (key) in fresh medium without RO-3306 or LPS; numbers along right margin indicate percent mitotic cells. Right, immunoprecipitation and immunoblot analysis of the interaction between endogenous NEK7 and NLRP3 in cells as at left. (d) ELISA of IL-1β (top) in supernatants of J774A.1 cells primed for 4 h with LPS alone (far left) or primed for 4 h with LPS and stimulated for 40 min with nigericin without arrest (green) or arrested with RO-03306 as in c, with no release from arrest (purple) or with release from arrest 1 h before (teal) or concurrent with (blue) stimulation with nigericin. Below, protocol: bar height indicates length of time cells were (black) or were not (gray) incubated with RO-3306, and time during which cells were treated with LPS and nigericin. (e) ELISA of IL-1β in supernatants of J774A.1 cells stably transfected with empty vector (EV) or with vector for the overexpression of wild-type NEK7 or NEK7(K64M) (key), primed with LPS and left unstimulated or stimulated with nigericin, and arrested with RO-03306 as in c or not, with release at the time nigericin was added, presented as quantification of IL-1β (left) or amount of IL-1β relative to that of cells treated with LPS plus nigericin, without RO-3306, set as 100% (right). *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001 (unpaired, two-tailed Student’s t-test). Data are representative of two independent experiments (mean and s.d. of biological triplicates in b,d,e).

Together the data reported above indicated that cells in interphase had a greater basal quantity of the NEK7-NLRP3 complex than did mitotic cells and that stimulation of the inflammasome increased the amount of NLRP3 bound to NEK7 and the amount of activated caspase-1 specifically in cells in interphase, which resulted in more production of IL-1β by cells in interphase than by mitotic cells. We also found that overexpression of NEK7 in J774A.1 cells enhanced NEK7-NLRP3 binding in mitotic cells primed with LPS and stimulated with nigericin compared with that of mitotic cells primed with LPS and stimulated with nigericin but lacking NEK7 overexpression (Fig. 7a). In addition, the production of IL-1β by mitotic J774A.1 cells was enhanced by overexpression of NEK7 or NEK7(K64M) (Fig. 7c). Our findings suggested that a limiting amount of cellular NEK7 might be available for either mitosis or inflammasome activation, but not both simultaneously.

We investigated whether component(s) of mitotic cell extracts activated the NLRP3 inflammasome in a NEK7-dependent manner. We found that the production of IL-1β induced by extracts of mitotic cells was similar for LPS-primed Nek7+/− macrophages and their Nek7+/+ counterparts and was dependent on the AIM2 inflammasome (Supplementary Fig. 7b and data not shown). Thus, the function of NEK7 in inhibiting simultaneous mitosis and inflammasome activation is probably not the prevention of aberrant activation of the inflammasome induced by nuclear activators exposed during breakdown of the nuclear membrane in mitosis.

DISCUSSION

NEK7, a member of the NIMA (‘never in mitosis gene a’)-related serine-threonine kinase family, has been linked to mitotic progression downstream of NEK9 (refs. 5–9). Depletion of NEK7 by RNA-mediated interference arrests cells in mitosis and induces apoptosis; these defects stem from impaired formation of mitotic spindles that might be secondary to deficiencies in microtubule organization7,8,10. If allowed to progress past the spindle-assembly checkpoint, cells depleted of NEK7 then arrest in cytokinesis8,10,11. Mouse embryonic fibroblasts derived from homozygous NEK7-null embryos display aneuploidy, polyploidy and a greater frequency of binuclear cells, which
confirms the proposal of a role for NEK7 in mitosis and cytokinesis\(^1\). Our findings that only 18.8\% of the expected number of Nek\(^{+/−}\)/Cu/Cu offspring were born from heterozygous crosses and that these mice displayed infertility, an abnormal gait and slight paralysis of the limbs support the proposal of an important but redundant role for NEK7 in mitosis. Here we established that NEK7, by direct interaction with NLRP3, was also a component of the NLRP3 inflammasome that contributed to its assembly in macrophages in response to priming with LPS and stimulation with nigericin, ATP or alum. The observation that the binding of NEK7 to NLRP3 required ROS, a mediator of various activators of NLRP3 (ref. 29), supported the proposal of a broad role for NEK7 in NLRP3 inflammasome activation.

Our findings suggested non-redundant functions for NEK7 in activation of the NLRP3 inflammasome and mitosis that could not occur simultaneously. In support of that hypothesis, activation of caspase-1 and production of IL-1\(\beta\) induced by LPS plus nigericin were robust during interphase but were much lower during mitosis, and overexpression of NEK7 partially restored the production of IL-1\(\beta\) by mitotic cells.

That last point, and the finding that priming with LPS plus stimulation with nigericin failed to increase the endogenous NEK7-NLRP3 interaction in mitotic cells, suggested that cellular amounts of NEK7 were limiting in the ‘choice’ between mitosis and inflammasome activation. The cellular importance of precluding activation of the NLRP3 inflammasome during mitosis is unknown. We hypothesized that this regulation might prevent aberrant activation of the NLRP3 inflammasome by putative nuclear activators exposed to the cytosol upon breakdown of the nuclear envelope during mitosis. However, extracts of mitotic cells induced similar production of IL-1\(\beta\) by LPS-primed Nek\(^{+/−}\)/macrophages and their Nek\(^{−/−}\) counterparts, which was dependent on the AIM2 inflammasome; this suggested that NLRP3 was not activated by nuclear components exposed during mitosis.

During mitosis, the nuclear membrane dismantles, vesicle trafficking halts, Golgi and endoplasmic reticulum membranes reorganize, actin and microtubule cytoskeletons undergo restructuring, chromosomes condense, and transcription and translation slow or stop\(^30\). These cellular changes might prohibit an NLRP3-dependent inflammatory response, which requires microtubule polymerization\(^4\), localization of NLRP3 and ASC at the perinuclear region\(^2,3\), secretion of IL-1\(\beta\) and IL-18 (ref. 31), and the induction of IL-1\(\beta\)- and IL-18-dependent transcriptional programs. IL-1\(\beta\) is known to induce the production of H\(_2\)O\(_2\) and oxygen radicals capable of damaging DNA\(^32,33\), which is not readily repaired during mitosis\(^34\). The phagocytic response also involves the generation of abundant ROS during the respiratory burst. Thus, dependence on NEK7 might have evolved as one of several proximal switches to avoid a futile or potentially damaging inflammatory response during cell division. In principle, if NEK7 is a switch between mitosis and the NLRP3 inflammasome response, the converse situation in which mitosis cannot be initiated in cells with activated NLRP3 inflammasomes should be true, possibly for similar reasons, but this has not been formally tested.

NEK7 bound directly to NLRP3, an interaction dependent on the LRR domain of NLRP3 and required for inflammasome assembly. Notably, several mutations encoding substitutions within the LRR domain of NLRP3 have been linked to neonatal onset multisystem inflammatory disease\(^24–26\). Our findings would suggest that the aberrant activation of NLRP3 inflammasomes in myeloid cells of patients with such mutations might stem from an increased association between NLRP3 and NEK7. Targeting this interaction might represent an alternative to neutralization of IL-1\(\beta\) for the treatment of NLRP3-mediated autoinflammatory diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank H. Zaki (University of Texas Southwestern Medical Center) for Nlrp3\(^{−/−}\) (B6.129S6-Nlrp3\(^{−/−}\)(C57Bl/6J)) mice; and F. Shao (National Institute of Biological Sciences, Beijing, China) for the Nlrc4 pneum. Supported by the US National Institutes of Health (U19 AI106027).

AUTHOR CONTRIBUTIONS

H.S and B.B designed the study and analyzed data, with suggestions from N.L.M. and K.J.U.; H.S., Y.W., L.S., D.C., V.V.K. and J.C.M. performed experiments; H.S. and Y.W. identified the Cuties phenotype; X.L., Z.X., M.T. and M.F. generated the Nek7-knockout mice; D.P., C.H.B., S.H., S.L., L.Sc, I.Q. and Q.S performed genome mapping and genotyping; H.S., J.R. and S.A. maintained the Cuties mice; H.S., P.J., E.M.Y.M. and B.B. edited the figures; and H.S., E.M.Y.M. and B.B. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
23. Pelegrin, P., Barroso-Gutierrez, C., & Surprenant, A. P2X7 receptor differentially couples to distinct release pathways for IL-1\(\beta\) in mouse macrophage. J. Immunol. 180, 7147–7157 (2008).

24. Matsubayashi, T., Sugiuwa, H., Arai, T., Oh-Ishi, T., & Inamo, Y. Anakinra therapy for CINCA syndrome with a novel mutation in exon 4 of the CIAS1 gene. Acta Paediatr. 95, 246–249 (2006).

25. Akserciévich, I., Remmers, E.F., Goldbach-Mansky, R., Reiff, A., & Kastner, D.L. Mutational analysis in neonatal-onset multisystem inflammatory disease: comment on the articles by Frenkel et al. and Saito et al. Arthritis Rheum. 54, 2703–2704 (2006).

26. Jesus, A.A. et al. Phenotype-genotype analysis of cryopyrin-associated periodic syndromes (CAPS): description of a rare non-exon 3 and a novel CIAS1 missense mutation. J. Clin. Immunol. 28, 134–138 (2008).

27. Kandli, M., Feige, E., Chen, A., Kiflin, G., & Motro, B. Isolation and characterization of two evolutionarily conserved murine kinases (Nek6 and Nek7) related to the fungal mitotic regulator, NIMA. Genomics 68, 187–196 (2000).

28. Vassilev, L.T. et al. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc. Natl. Acad. Sci. USA 103, 10660–10665 (2006).

29. Weinberg, S.E., Sena, L.A., & Chandel, N.S. Mitochondria in the regulation of innate and adaptive immunity. Immunity 42, 406–417 (2015).

30. Cooper, G.M. in The Cell: A Molecular Approach (Sinauer Associates, Sunderland, Massachusetts, 2000).

31. Eder, C. Mechanisms of interleukin-1\(\beta\) release. Immunobiology 214, 543–553 (2009).

32. Li, Q. & Engelhardt, J.F. Interleukin-1beta induction of NF\(\kappa\)B is partially regulated by H2O2-mediated activation of NF\(\kappa\)B-inducing kinase. J. Biol. Chem. 281, 1495–1505 (2006).

33. Bertram, C. & Hass, R. Cellular responses to reactive oxygen species-induced DNA damage and aging. Biol. Chem. 389, 211–220 (2008).

34. Orthwein, A. et al. Mitosis inhibits DNA double-strand break repair to guard against telomere fusions. Science 344, 189–193 (2014).
ONLINE METHODS

Mice. Eight- to ten-week old male and female mice (Mus musculus) on a pure C57BL/6J background were used in experiments. Male C57BL/6J mice purchased from The Jackson Laboratory were mutagenized with N-ethyl-N-nitrosourea as described.13 Mutagenized G0 males were bred to C57BL/6J females, and the resulting G1 males were crossed to C57BL/6J females to produce G2 mice. G2 females were backcrossed to their G1 sires to yield G3 mice, which were screened for phenotype. Whole-exome sequencing and mapping were performed as described.13 Nek7Gurate (https://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pkp=757), Nlrp3^D946G (Nlrp3^jklik) (https://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pkp=1333) and Nek6 (https://mutagenetix.utsouthwestern.edu/incidental/incidental_rec.cfm?id=7622&rin=1&r=1&rl=10&so=ge&ac=1&r0=0&nr=100&gsm=nek6) mutant strains were generated by mutagenesis with N-ethyl-N-nitrosourea and are described online.

All experimental procedures using mice were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center and were conducted in accordance with institutionally approved protocols and guidelines for animal care and use. All mice were maintained at the University of Texas Southwestern Medical Center in accordance with institutionally approved protocols. Animals were to be excluded from analysis only if they displayed obvious illness or death; these conditions were not observed and no animals were excluded. No randomization of the allocation of samples or animals to experimental groups was performed.

Bone marrow chimeras. Recipient wild-type (CD45.1+) mice were lethally irradiated with 950 rads via a 137Cs source and, 2–3 h later, were given intravenous injection of 3 × 10^6 bone marrow cells derived from the tibia and femur of the respective donors (CD45.2+). 7 weeks after being engrafted, the chimeras were assessed by in vivo challenge with MSU.

Generation of Nek7−/− mice via CRISPR. Female C57BL/6J mice were superovulated by injection of 6.5 U pregnant mare serum gonadotropin (Millipore), followed by injection of 6.5 U human chorionic gonadotropin (Sigma-Aldrich) 48 h later. The superovulated mice were subsequently mated overnight with C57BL/6J male mice. The following day, fertilized eggs were collected from the oviducts and in vitro–transcribed Cas9 mRNA (50 ng/µl) and Nek7 small base-pairing guide RNA (50 ng/µl; 5′-CTGTTAATTTAATACGGT3′) were injected into the cytoplasm or pronucleus of the embryos. The injected embryos were cultured in M16 medium (Sigma-Aldrich) at 37 °C in 95% air and 5% CO2. For the production of mutant mice, two-cell stage embryos were transferred into the ampulla of the oviduct (10–20 embryos per oviduct) of pseudopregnant Hsd:ICR (CD-1) female mice (Harlan Laboratories). Chimeric mutant mice were first crossed with C57BL/6J mice and their offspring were intercrossed for the generation of Nek7−/− mice. Nek7−/− mice have a four–base pair net deletion in Nek7 exon 2 (five–base pair deletion (in italics below) and one–base pair insertion (underlined below): TTTCAGCACAGATTTAATTTAATA). The Nek7−/− mice were genotyped by capillary sequencing with 5′-CCGGAGAAGTGGAAATGGTGT-3′ and 5′-CCA GACTATCAGTAACCCTCAAAGCC-3′ as the PCR primers and 5′-TATG TGAACATTACACAGCCTTG-3′ as the sequencing primer.

Reagents. Ultra-pure LPS, MALP-2 and R848 were obtained from Enzo Life Sciences; for stimulation of human monocytes, LPS (O111:B4) was from List Biologicals; for stimulation of mouse macrophages, E. coli lipopolysaccharide and ODN 2006 were from InvivoGen; LPS and ODN 2006 were used in culture medium at a final concentration of 100 µg/ml; or for 16 h with E. coli, (11775; American Type Culture Collection) or C. rodentium (51459; American Type Culture Collection). Poly(dA:dT) (4 µg/ml) (8 h of incubation) and flagellin (500 ng/ml) (2 h of incubation) were made into a complex with Lipofectamine 2000 and then were transfected into cells according to the manufacturer’s instructions (Life Technologies). HEK293T cells were washed with cold PBS and then were lysed in homogenization buffer (10 mM Tris-HCl, pH 7.5, 2 mM MgCl2 and 250 mM sucrose) by 20 strokes with a Dounce homogenizer. The homogenate was centrifuged at 5,000g for 10 min, and the supernatant was used as cytosol extract. Extracts of mitotic cells were prepared from HEK293T cells treated overnight with RO-3306 in the culture medium, followed by 1 h of release in fresh medium. The cells were washed with cold PBS and then were lysed in homogenization buffer with gentle sonication. Following priming with LPS, cells were transfected for 14 h with the extract (1.5 µg per 1 × 10^6 cells) through the use of Lipofectamine 2000.

Flow cytometry. Blood was collected in Minicollect Tubes (Mercedes Medical) and centrifuged at 700g for separation of serum, and red blood cells remaining in the serum were lysed (eBiologie) before staining of cells of the immune system and flow cytometry. Cells were incubated with monoclonal antibody to CD61/32 and were labeled for 1 h at 4 °C using fluorochrome-conjugated monoclonal antibodies to mouse CD3, CD4, CD8a, B220, NK1.1, F4/80 and CD11b (all antibodies identified above). Data were acquired using an LSRFortessa Cell Analyzer (BD Biosciences).

Isolation and culture of peritoneal macrophages, bone marrow–derived macrophages, bone marrow–derived dendritic cell. Macrophages were elicited by intraperitoneal injection of 2 ml BBL thioglycollate medium, brewer modified (4%; BD Biosciences), they were recovered 4 d later by peritoneal lavage with 5 ml phosphate-buffered saline (PBS). The peritoneal macrophages were cultured at 37 °C in 95% air and 5% CO2 in DMEM cell culture medium (DMEM containing 10% FBS, 1% penicillin and streptomycin (Life Technologies)). For determination of the ability of macrophages to induce CD11b (all antibodies identified above). Data were acquired using an LSRFortessa Cell Analyzer (BD Biosciences).

Measurement of cytokine production. Cells were seeded onto 96-well plates at a density of 1 × 10^5 cells per well and then were stimulated as follows: for 4 h with LPS (10 ng/ml), MALP-2 (400 pg/ml) or R848 (20 ng/ml); for 1 h with nigericin (10 µg/ml) or ATP (5 mM); for 8 h with alum (400 µg/ml); or for 16 h with E. coli, (11775; American Type Culture Collection) or C. rodentium (51459; American Type Culture Collection). Poly(dA:dT) (4 µg/ml) (8 h of incubation) and flagellin (500 ng/ml) (2 h of incubation) were made into a complex with Lipofectamine 2000 and then were transfected into cells according to the manufacturer’s instructions (Life Technologies). HEK293T cells were washed with cold PBS and then were lysed in homogenization buffer (10 mM Tris-HCl, pH 7.5, 2 mM MgCl2 and 250 mM sucrose) by 20 strokes with a Dounce homogenizer. The homogenate was centrifuged at 5,000g for 10 min, and the supernatant was used as cytosol extract. Extracts of mitotic cells were prepared from HEK293T cells treated overnight with RO-3306 in the culture medium, followed by 1 h of release in fresh medium. The cells were washed with cold PBS and then were lysed in homogenization buffer with gentle sonication. Following priming with LPS, cells were transfected for 14 h with the extract (1.5 µg per 1 × 10^6 cells) through the use of Lipofectamine 2000.

DOI:10.1038/ni.3333
Primary human monocytes were stimulated for 4 h with LPS (1 ng/ml) and for 1 h nigericin (10 µg/ml). Cytokine concentrations in the supernatants were measured with ELISA kits for human IL-1β, IL-6, mouse IL-1β, IL-18, IL-6 and tumor-necrosis factor (eBioscience).

Lactate dehydrogenase assay. The release of lactate dehydrogenase into the culture medium was determined by an LDH cytotoxicity assay kit according to the manufacturer's instructions (Pierce).

Measurement of mitochondrial ROS. Peritoneal macrophages were stained with Mitosox for 30 min in accordance with the manufacturer's instructions (Life Technologies). The cells were suspended in 1 % fetal bovine serum (FBS) in PBS and then were analyzed by flow cytometry with an LSRFortress Cell Analyzer.

Measurement of intracellular CAMP. 1 × 10⁶ peritoneal macrophages were given the appropriate treatment(s). The cells were then resuspended in cold 0.1 N HCl/Cell Lysis Buffer (provided by the kit noted below). The cAMP in the cell lysates was assayed with a Mouse/Rat cAMP Parameter Assay Kit according to the manufacturer's instructions (R&D Systems).

Calcium measurement. Peritoneal macrophages were plated on four-chambered coverglass dishes (Fisher Scientific) at a density of 4 × 10⁵ cells per well and incubated with Fluor-4/AM (Life Technologies). Images of untreated cells were acquired (time 0), then cells were treated with 5 mM ATP in DMEM cell culture medium and were imaged every 15 s for 30 min. Images were acquired with an Andor Confocal Spinning Disk microscope with the 488-nm laser and emission in the range of 500–560 nm. Images were analyzed with ImageJ 1.47v software. The absolute intensity for all cells in a field at different time points was obtained and results were normalized to those at time 0 for calculation of the increase in intensity. Data are presented as the mean fluorescence intensity of all cells in a field (about 20 cells per field) relative to that at time 0 and are representative of four independent fields.

ASC oligomerization assay. Peritoneal macrophages were lysed with TBS buffer (50 mM Tris–HCl (pH 7.4) and 150 mM NaCl) containing 0.5 % Triton X-100, EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). The lysates were centrifuged at 6,000g for 15 min at 4 °C, and the pellets and supernatants were used as the Triton-insoluble fractions and Triton-soluble fractions, respectively. For the detection of ASC oligomerization, the Triton-insoluble pellets were washed twice with TBS buffer and then were resuspended in 300 µl TBS buffer. The resuspended pellets underwent crosslinking for 30 min at 37 °C with 2 mM disuccinimidyl suberate (Pierce) and then were centrifuged for 15 min at 6,000g. The pellets were dissolved in SDS sample buffer.

siRNA-mediated interference. J774A.1 or THP-1 cells (American Type Culture Collection) in 12-well cell culture plates were transfected with 40 pM small interfering RNA through the use of Lipofectamine RNAiMAX (Life Technologies). After incubation for 48 h, the cells were washed and used in experiments. The siRNA specific for mouse or human NEK7 mRNA (SMART pool) and the non-targeting siRNA were from Dharmacon (GE Healthcare).

Isolation and culture of human monocytes and short hairpin RNA–mediated interference. Lentivirus encoding red fluorescent protein (RFP) were obtained from Dharmacon (GE Healthcare). Monocytes were isolated and short hairpin RNA specific for human NEK7 mRNA (sequence 3, GAGGCTAATTCCTGAAAGA) and the non-targeting control were prepared with NP-40 lysis buffer and gentle sonication. Soluble lysate (200 ng/well) and mouse inflammasome components ASC (20 ng/well), pro-caspase-1 (100 ng/well) and NLRP3 (200 ng/well) through the use of Lipofectamine 2000. These modified cells were then co-transfected with plasmid encoding mouse NEK7 or specific siRNA to knock down endogenous NEK7. The total amount of DNA was adjusted to a concentration of 1 µg per well through the use of empty vector. The cells were washed with culture medium 36 h after transfection and were further incubated for 12 h. Cell culture medium was collected and IL-1β maturation was assessed by immunoblot analysis by standard techniques (antibodies identified above).

Reconstruction of the inflammasome system in HEK293T cells. HEK293T cells were plated in 24-well microplates at a density of 2 × 10⁵ cells per well and were incubated overnight. The cells were transfected with plasmids expressing Flag-tagged mouse pro–IL-1β (200 ng/well) and mouse inflammasome components ASC (20 ng/well), pro-caspase-1 (100 ng/well) and NLRP3 (200 ng/well) through the use of Lipofectamine 2000. These modified cells were then co-transfected with plasmid encoding mouse NEK7 or specific siRNA to knock down endogenous NEK7. The total amount of DNA was adjusted to a concentration of 1 µg per well through the use of empty vector. The cells were washed with culture medium 36 h after transfection and were further incubated for 12 h. Cell culture medium was collected and IL-1β maturation was assessed by immunoblot analysis by standard techniques (antibodies identified above).

Immunoprecipitation. At 24 h after transient transfection as described above, HEK293T cells were lysed in cold NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl, pH 7.4, and 150 mM NaCl) supplemented with Roche Complete Protease Inhibitor. Flag-tagged proteins were immunoprecipitated with Anti-FLAG M2 Magnetic Beads (Sigma–Aldrich). For the endogenous interaction assay, macrophages or J774A.1 cells were lysed with NP-40 lysis buffer with Complete Protease Inhibitor. The cell lysates were incubated overnight at 4 °C with the appropriate antibodies (identified above) and Protein G Mag Sepharose (GE Healthcare). For the NLRP3-ASC interaction assay, macrophages or J774A.1 cells were lysed with NP-40 lysis buffer and gentle sonication.

Gel-filtration chromatography. Fresh lysates from mouse macrophages were prepared with NP-40 lysis buffer and gentle sonication. Soluble lysate (0.6 mg of total protein) was run on a gel-filtration chromatography column (Superdex 200 Increase 10/300 GL, GE Healthcare) in NP-40 lysis buffer with 1 mM Dithiothreitol. 1 ml fractions were collected and 1.5 % of each fraction subjected to immunoblot analysis.

In vitro kinase assay. The kinase assay was performed with 10 ng recombinant MBP-NEK or MBP-NEK7(K64M) and 15 µg β-casein (Sigma–Aldrich), as a substrate, with a Universal Kinase Activity Kit according to the manufacturer's instructions (R&D Systems). MBP-NEK and MBP-NEK7(K64M) were expressed in E.coli and were purified with amylose beads.

Bone marrow progenitor colony-forming unit assay. Bone marrow cells were collected from tibiae and femurs and were treated with red blood cell lysis buffer (Sigma–Aldrich). 1 × 10⁵ cells and 50 ng/ml mouse macrophage colony-stimulating factor (eBioscience) were added to Mouse Methylocellulose Base Media according to the manufacturer's instructions (R&D Systems). Individual colonies were counted 8 days later.

Apoptosis analysis. Peritoneal macrophages were plated in 96-well, clear, flat-bottomed, ultra-low–attachment microplates (Corning). The cells underwent the appropriate treatment(s) and then were stained with annexin V and 7-amino-actinomycin D according to the manufacturer's instructions (BD Biosciences).

In vivo challenge with MSU and IL-1β. Mice were challenged by intraperitoneal injection of MSU (1 mg) or one dose of active recombinant mouse IL-1β in sterile PBS (50 ng/100 µl). After 6 h, the mice were euthanized and
peritoneal cavities underwent lavage with 5 ml PBS. PECs were collected and analyzed by flow cytometry with an LSRFortessa Cell Analyzer. For ELISA of IL-1β, the peritoneal fluid was concentrated with Amicon Ultra 10K filtration units (Millipore) and then was processed.

Induction of EAE and flow cytometry analyzing leukocyte infiltration in the CNS. Recombinant human myelin oligodendrocyte glycoprotein (MOG) (amino acids 1–125) was generated as described. EAE was induced by subcutaneous immunization of mice at four sites on the back with 100 μg of recombinant human MOG emulsified in complete Freund’s adjuvant (Difco) containing 5 mg/ml of mycobacteria (BD Biosciences). On days 0 and 2, mice were given intraperitoneal injection of 300 ng pertussis toxin (List Biological Laboratories). Clinical disease was assessed as follows: 0, no disease; 1, loss of tail tone; 2, weakness of hind limbs; 3, partial hind limb paralysis; 4, total hind limb paralysis with or without front limb paralysis; 5, moribund or death.

On day 21 after immunization with recombinant human MOG, mice were euthanized and then were perfused via the left ventricle with cold PBS supplemented with 10 U/ml heparin (Fisher Scientific). Spinal cords were collected from the perfused mice. Tissues were pressed through a 70-μm cell strainer into RPMI-1640 medium (Corning). Spinal cord cells were pelleted by centrifugation at 390 g for 10 min at 4 °C. Cell pellets were resuspended in 30% Percoll (GE Healthcare) and were centrifuged at 390g for 20 min at room temperature with the brake off. The cell pellets were collected and washed with RPMI-1640 medium and were counted. For staining with the ten-color survey panel, monoclonal antibodies to the following mouse molecules were used: CD3ε (145-2C11), CD4 (RM4-5), B220 (RA3-6B2), CD19 (1D3) and CD11b (ICRF44) (all from eBioscience); TCR ε (H57-597), Gr1 (RB6-8C5) and NK1.1 (PK136) (all from BD Biosciences); and CD45 (30-F11; BioLegend). Cell viability dye (Tonbo Biosciences) was used to distinguish viable cells from dead cells.

Infection and treatments of J774A.1 cells. J774A.1 cells were infected with retrovirus encoding mouse NEK7, NEK7(K64M) or empty vector (pMXs-IRES-Puro, Cell BioLabs). 48 h after the infection, the cells were selected and cultured with 2 μg/ml puromycin (InvivoGen).

LPS-primed J774A.1 cells were treated for 2 h with colchicine (10 μM), nocodazole (10 μM), cytochalasin D (5 μM) or ciliobrevin D (10 μM), followed by stimulation for 1 h with nigericin. LPS-primed J774A.1 cells were treated for 1 h with ATP and N-acetylcysteine. For treatment with calf intestinal alkaline phosphatase (CIP), 100 mg cell lysate was incubated for 1 h at 37 °C with 50 units of CIP (New England Biolabs). Immunoprecipitation was performed as described above. For treatment with CIP, the immunoprecipitated complex was further incubated for 1 h at 37 °C with 50 units of CIP in a reaction volume of 50 μl. Immunoprecipitates were separated by standard SDS-PAGE or by Phos-tag SDS-PAGE (Wako Chemicals) for the separation of phosphorylated proteins according to their degree of phosphorylation.

Intracellular staining of activated caspase-1. For the measurement of intracellular activated caspase-1, a FLICA (fluorescence-labeled inhibitor of caspasess) probe specific for active caspase-1 (ImmunoChemistry Technologies) was used. The probe is cell permeable and covalently binds to the active form of caspase-1, which is retained within the cell while unbound probe diffuses out of the cell and is washed away. After priming of cells with LPS, the FAM–FLICA–caspase-1 probe was added directly to the cell culture medium together with nigericin, followed by incubation for 1 h at 37 °C, then cells were washed twice with the wash buffer supplied together with FLICA probe (ImmunoChemistry Technologies). Cells were fixed and stained for labeling of mitotic cells before analysis by flow cytometry with an LSRFortessa Cell Analyzer.

Staining and sorting of mitotic cells. Where indicated, cells were treated for at least 20 h with RO-3306 (10 μM) in the culture medium for synchronization of cells at the G2-M phase ‘border’, then cells were released from the RO-3306–imposed cell-cycle block for various time periods by replacement of the medium with fresh culture medium.

Cells were collected and then were fixed for 20 min at 4 °C in Fixation/Permeabilization solution (BD Biosciences), followed by staining with Alexa Fluor 647–conjugated antibody to histone H3 phosphorylated at Ser28 (HTA28; BioLegend) in 50 μl of BD Perm/Wash buffer (BD Biosciences). Cells were analyzed by flow cytometry or were sorted before immunoprecipitation and immunoblot analysis.

Statistical analysis. Comparisons of differences were between two unpaired experimental groups in all cases. An unpaired t-test (Student’s t-test) is appropriate and was used for such comparisons. The phenotypic performance of mice (C57BL/6J) and primary cells of these mice is expected to follow a normal distribution, as has been observed in large data sets from numerous phenotypic screens conducted by our group. Variation within each data set obtained by measurements from mice or primary cells was assumed to be similar between genotypes since all strains were generated and maintained on the same pure inbred background (C57BL/6J); experimental assessment of variance was not performed.

The statistical significance of differences between experimental groups was determined with GraphPad Prism 6 software and the Student’s t test.