An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome

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Inflammasomes are innate immune sensors that respond to pathogen- and damage-associated signals with caspase-1 activation, interleukin (IL)-1β and IL-18 secretion, and macrophage pyroptosis. The discovery that dominant gain-of-function mutations in NLPR3 cause the cryopyrin-associated periodic syndromes (CAPS) and trigger spontaneous inflammasome activation and IL-1β oversecretion led to successful treatment with IL-1–blocking agents. Herein we report a de novo missense mutation (c.1009A>T, encoding p.Thr337Ser) affecting the nucleotide-binding domain of the inflammasome component NLRC4 that causes early-onset recurrent fever flares and macrophage activation syndrome (MAS). Functional analyses demonstrated spontaneous inflammasome formation and production of the inflammasome-dependent cytokines IL-1β and IL-18, with the latter exceeding the levels seen in CAPS. The NLRC4 mutation caused constitutive caspase-1 cleavage in cells transduced with mutant NLRC4 and increased production of IL-18 in both patient-derived and mutant NLRC4–transduced macrophages. Thus, we describe a new monoallelic inflammasome defect that expands the monoogenic autoinflammatory disease spectrum to include MAS and suggests new targets for therapy.

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In our protocol investigating and treating individuals with early-onset autoinflammatory diseases (NCT00059748), we evaluated a 7-year-old female of European ancestry with recurrent episodes of fever, malaise, splenomegaly, vomiting, loose stools with mild duodenitis, and occasional rash beginning at 6 months of age (Fig. 1a–d). Fever episodes were initially attributed to recurrent viral infections, but prolonged flares requiring corticosteroid and colchicine treatment and negative evaluations for infection, immunodeficiency and malignancy suggested an autoinflammatory syndrome (Supplementary Note and data not shown). Over time, chronic inflammation and corticosteroid dependence contributed to poor growth (Supplementary Fig. 1a). On laboratory evaluation, the combination of elevated inflammatory markers, chronic anemia, transaminitis, hypertriglyceridemia, hyperferritinemia, leucopenia and thrombocytopenia with severe flares was not consistent with CAPS but suggestive of MAS (Fig. 1e and Supplementary Fig. 1b).

MAS and the related entity hemophagocytic lymphohistiocytosis (HLH) are life-threatening systemic immune-dysregulatory conditions associated with uncontrolled macrophage activation and hemophagocytosis. MAS and HLH flares present with fevers, pancytopenia, elevated triglyceride levels, impaired natural killer (NK) cell killing and very high serum ferritin levels. If left untreated, these flares can progress to coagulopathy, organ failure and death. MAS is known complication of some rheumatic diseases (including systemic lupus erythematosus, adult-onset Still’s disease (AOSD) and systemic juvenile idiopathic arthritis (JIA)) but is rarely observed in CAPS or other periodic fever syndromes. MAS and HLH can also be triggered by infections (for example, by Epstein-Barr virus) and malignancies. Insights into the pathogenesis of familial HLH came from genetic discoveries that loss-of-function mutations in PRF1 (encoding perforin) or other genes necessary for perforin- and/or granzyme-mediated killing result in the unchecked activation and survival of macrophages and dendritic cells. These associations have shaped the concept that impaired cytotoxic cell killing drives the pathogenesis of HLH and possibly MAS.

NK cell function was normal in our patient, and genetic testing for HLH and for periodic fever syndromes was negative (Supplementary Note). To pursue a molecular diagnosis, she underwent whole-exome sequencing, which identified a heterozygous de novo mutation resulting in a p.Thr337Ser substitution in a highly conserved region of the NLRC4 nucleotide-binding domain (NBD) (Fig. 2a,b and Supplementary Fig. 2a,b). The variant was confirmed by Sanger sequencing and predicted to be pathogenic on the basis of conservation, pathogenicity prediction packages and absence from the Exome Sequencing Project (ESP) database of more than 6,500 control alleles and an in-house collection of >150 exomes (Supplementary Fig. 2b–d). Conformational analysis based on the crystal structure of the highly homologous mouse NLRC4 protein (recently solved by Hu et al.) suggested that this mutation might destabilize helical domain 1 interactions with NBD residues 170 and/or 173 or might directly affect ADP binding, any of which might be essential for maintaining NLRC4 in an autoinhibited state.
Figure 2 The NLRC4 mutation occurs de novo and affects a highly conserved area of the NBD. (a) Chromosomal location, adjacent exons and exon structure of the NLRC4 gene and domain structure of the NLRC4 protein. (b) Pedigree showing a de novo heterozygous mutation. The affected individual is denoted by a filled circle. WT, wild type. (c) Location of the amino acid substitution from threonine to serine at position 337 and R-group interactions with adjacent residues in the NBD that are predicted to be important for stabilizing ADP binding (on the basis of the crystal structure of the mouse protein). NBD, nucleotide-binding domain; WHD, winged-helix domain; HD, helical domain; LRR, leucine-rich repeat domain. The green dashed line represents predicted side chain interactions.

conformation (Fig. 2c). A nearby heterozygous NLRC4 mutation, encoding p.Val341Ala, has been reported by Romberg et al. to present similarly as recurrent MAS.

We hypothesized that the identified mutation might result in increased NLRC4 inflammasome activity and NLRC4-mediated MAS (which we subsequently refer to as NLRC4-MAS). Corroborating this hypothesis, we found a cluster of serum cytokines at elevated levels in NLRC4-MAS but not in controls or patients with NOMID, including the inflammasome-activated cytokine IL-18 (8,316–17,355 pg/ml in NLRC4-MAS, 102–1,281 pg/ml in NOMID and 56–105 pg/ml in controls; Fig. 3a). Comparably high levels of serum IL-18 have also been associated with MAS in systemic JIA (AOSD), infection and XIAP deficiency. Other serum markers that clustered distinctly in NLRC4-MAS included macrophage-stimulating cytokines (M-CSF and IL-12p40; ref. 28), apoptotic factors (TRAIL and LTα), chemokines (CCL7, CXCL12 and IL-16) and hematopoietic growth factors (SCF and IL-3) (Fig. 3a and Supplementary Fig. 3). Transcriptome analyses comparing the patient NLRC4-MAS to patients with NOMID and healthy controls similarly showed upregulation of genes associated with apoptosis and dysregulation of genes associated with macrophage activation (Supplementary Fig. 4 and Supplementary Table 1), particularly in an NLRC4-MAS disease flare sample. These findings are consistent with macrophage stimulation and activation with the upregulation of apoptosis and hematopoiesis. We also found the calgranulins S100A8, S100A9 and S100A12 were among the most highly upregulated genes during an NLRC4-MAS flare but were minimally upregulated in active NOMID (Fig. 3b and Supplementary Table 2). Increased calgranulin expression was not explained by an increased proportion of peripheral blood granulocytes, which constitutively express calgranulins at high levels (Supplementary Fig. 5). When calgranulins are released from activated monocytes and neutrophils, they act as damage-associated signals to drive proinflammatory responses. They are particularly elevated in sepsis, systemic JIA and AOSD, and familial Mediterranean fever but not in CAPS, suggesting that induction of the calgranulin damage response might help differentiate NLRC4-MAS from NLRC4-driven inflammation.

Because NLRP3 mutations lead to increased inflammasome activation and IL-1β and IL-18 secretion from monocytes and macrophages, we assessed these responses in cells derived from the patient with NLRC4-MAS. Although ATP is a canonical trigger of the NLRP3 inflammasome, studies in mice suggest that NLRC4 inflammasome activation is triggered by cytosolic flagellin. Unlike Toll-like receptor 5 (TLR5), which recognizes flagellin at the cell surface, the NLRC4 inflammasome recognizes a distinct region of flagellin that has been injected through a bacterial secretion system (as seen in Salmonella typhimurium or Legionella pneumophila infection). We assessed spontaneous and stimulated responses in primary monocytes and monocyte-derived macrophages after priming with lipopolysaccharide (LPS) and stimulation with triggers for NLRP3 (ATP) and NLRC4 (intracellular flagellin, IC-FLA) inflammasome activation.

As expected, monocytes from the patient with NLRC4-MAS and from a patient with NOMID (with an NLRP3 mutation encoding p.Gly569Arg) produced significantly more IL-1β and IL-18 with all stimuli than healthy controls, but no difference in IL-1β secretion was seen between NOMID and NLRC4-MAS monocytes (Fig. 4a). However, NLRC4-MAS macrophages secreted more IL-1β than NOMID macrophages. Interestingly, IL-18 was constitutively secreted from NLRC4-MAS monocytes and macrophages in contrast to comparable cells from the patient with NOMID and the healthy controls (Fig. 4a). Upon stimulation, IL-18 levels were not significantly further upregulated in monocytes, but they were significantly upregulated in stimulated macrophages. IL-18 levels were four to five times higher in stimulated macrophages than in monocytes, pointing to activated macrophages as a key source of IL-18 (Fig. 4a and Supplementary Fig. 6). IC-FLA–induced cytokine production was sustained with prolonged stimulation, suggesting that the mutation affects both the kinetics and amplitude of the cytokine response (Supplementary Fig. 7). Production of the inflammasome-independent cytokines IL-6, tumor necrosis factor (TNF)-α and IL-10 was elevated in NLRC4-MAS monocytes in comparison to control and NOMID cells, particularly before the initiation of IL-1–blocking therapy (Supplementary Figs. 6 and 8). However, these cytokines were not overproduced by matched monocyte-derived macrophages from the patient with NLRC4-MAS (Fig. 4a and Supplementary Figs. 6–8). Together, these results suggest increased responsiveness of NLRC4-MAS monocytes...
but specific overproduction of IL-1β and IL-18 by NLRC4-MAS macrophages and differential roles for the NLRP3 and NLRC4 inflammasomes in inducing IL-18 production and secretion. Inflammasome activation also results in inflammatory cell death termed pyroptosis34. To evaluate spontaneous and stimulated cell death, human monocyte-derived macrophages were cultured with or without IC-FLA stimulation and monitored for lactate dehydrogenase (LDH) release. Spontaneous cell death was significantly higher in NLRC4-MAS macrophages than in macrophages from healthy controls or patients with NOMID (Fig. 4b). Similarly, the incidence of cell death specifically attributable to IC-FLA stimulation was substantially higher in the NLRC4-MAS macrophages than in controls (Fig. 4c).

Inflammasome activation triggers the oligomerization of apoptosis-associated speck-like protein with CARD domain (ASC; also known as PYCARD) into aggregates that are necessary for caspase-1 activation and cytokine production1 in a process that can be visualized by fluorescence microscopy35. We assessed spontaneous and stimulated ASC aggregation in NLRC4-MAS and control monocyte-derived macrophages. NLRC4-MAS macrophages displayed spontaneous ASC aggregation that was not substantially altered by LPS and/or IC-FLA stimulation, whereas control macrophages showed stimulation-dependent ASC aggregation (Fig. 4d,e). These results corroborate constitutive inflammasome activation in NLRC4-MAS macrophages.

To investigate the activating effect of the NLRC4 mutation, we generated stable transductants of THP1 myelomonocytic cells that expressed either wild-type NLRC4 (WT), Thr337Ser mutant NLRC4 (T337S) or empty vector (EV) and assessed inflammasome activity. Expression of the transgene, as reported by GFP expression, was consistently lower in T337S cells than in WT cells, despite cells being infected with up to 200-fold more mutant than wild-type virus, suggesting a proliferative disadvantage for T337S cells (Fig. 5a and Supplementary Fig. 9). Despite lower NLRC4 protein expression, we measured increased spontaneous caspase-1 cleavage in T337S cells (Fig. 5a). Macrophages derived from T337S cells also showed enhanced secretion of IL-1β (T337S cells had 5.5- to 10.6-fold higher levels than EV cells, whereas WT cells had 2.4- to 5.9-fold higher levels than EV cells; P = 0.01) and IL-18 (T337S cells had 5.2- to 11.4-fold higher levels than EV cells, whereas WT cells had 2.1- to 6.7-fold higher levels than EV cells; P = 0.03) (Fig. 5b). These findings support a gain-of-function activity for the NLRC4 mutation through constitutive caspase-1 activation.

Given the successful use of IL-1–blocking strategies in NLRP3-mediated diseases3, the patient with NLRC4-MAS began treatment with recombinant IL-1 receptor antagonist (anakinra). The flare frequency, C-reactive protein levels, splenomegaly and prednisone dose were substantially reduced after the initiation of IL-1–blocking therapy (Fig. 6a, Supplementary Fig. 1b, and Supplementary Note). After 7 months of treatment, the patient had no fevers or clinical flares and had been weaned off corticosteroids and colchicine (Fig. 6a). She had no treatment-related adverse events but had transient transaminits and elevated lactate dehydrogenase (LDH) and ferritin levels after a brief viral illness. Consistent with ongoing subclinical inflammation, her pattern of serum cytokinemia had not normalized after treatment with anakinra (Figs. 3 and 6b, and Supplementary Fig. 3), and cytokine production by her monocytes and macrophages did not change with treatment (Fig. 4 and Supplementary Figs. 6 and 7).
the NBD of NLRC4 to the clinical and immunological phenotype of MAS. Mutations in NLRP3 that affect the NBD cause constitutive inflammasome activity and CAPS, and gain-of-function mutations in NOD2 (also known as CARD15) that map to the NBD cause Blau syndrome\(^1\). The presence of similar activating mutations in these NLRs, the strong across-species conservation of the NBD domain and the role of the NBD in mediating autoinhibition\(^6\) support constitutive NLR oligomerization as a key mechanism in these three diseases. Unlike in individuals with CAPS or NOMID, the NLRC4 mutation in this patient causes very high circulating and stimulated levels of IL-18, similar to the levels seen in other MAS-prone conditions\(^25\)–\(^27,36\). The factors and molecular mechanisms that differentially regulate NLRC4 versus NLRP3 inflammasome activation are largely unknown and require further study. In addition, IL-18 production by non-hematopoietic cells (including osteoblasts, adrenal cortex and intestinal epithelia\(^37\)) might incrementally contribute to the constitutively high serum IL-18 levels in NLRC4-MAS in comparison to CAPS.

**Figure 4** NLRC4-MAS monocytes and macrophages have increased inflammasome-related cytokine secretion, cell death and ASC aggregate formation. (a) Monocytes and monocyte-derived macrophages from healthy controls (Ctrl) and from patients with NLRC4-MAS and NOMID were primed with LPS and stimulated with purified flagellin (FLA), liposomal flagellin (IC-FLA) or ATP. Secreted cytokines were measured by Luminex. Bars represent the mean and s.d. of technical duplicates. Monocytes and macrophages represent matched samples. In LPS-primed cells, the addition of ATP did not alter cytokine production (data not shown). (b,c) LDH release as a marker of cell death, presented as the percentage of maximum lysis induced by 0.8% Triton X-100, was measured in macrophages after resting them for 6 h in serum-free medium alone (b) or after stimulation (c) with varying doses of IC-FLA for 3 h (top) or with 5 µg/ml IC-FLA for the indicated time periods (bottom). Cell death assays were performed in biological quadruplicate. The mean and s.d. are representative of two independent experiments. Statistical comparisons were evaluated by one-way ANOVA with Tukey’s post-test. *P < 0.05, **P < 0.01, ***P < 0.0001; NS, not significant. Comparisons between NLRC4-MAS and NOMID are depicted. (d,e) Macrophages were stimulated as indicated and imaged by confocal immunofluorescence microscopy. Red, ASC; green, caspase-1; blue, nuclei. Representative images (d) with a detail of a representative ASC aggregate (arrows and inset) were taken, and ASC aggregate formation (e) was quantified. Scale bars, 10 µm.

All samples from patients with NLRC4-MAS and NOMID (a–e) were obtained after IL-1 receptor antagonist treatment for at least 3 months.

**Figure 5** Cells transduced with virus expressing mutant NLRC4 exhibit spontaneous inflammasome activity. THP1 monocytes were stably transduced with empty retrovirus (EV) or retrovirus carrying wild-type or Thr337Ser mutant NLRC4 and were differentiated into macrophages (Online Methods). (a) Immunoblots of whole-cell lysates from transduced THP1 cells. (b) Cytokine concentrations were measured by ELISA in culture supernatants from THP1 cells transduced with construct for wild-type or mutant NLRC4 and differentiated into macrophages with low-dose phorbol 12-myristate 13-acetate (PMA) in technical triplicates (Online Methods). The values expressed represent the average concentration of the technical replicates for macrophages expressing wild-type or Thr337Ser NLRC4 divided by the average concentration of the technical replicates for macrophages transduced with empty viral vector within the same experiment. Each data point represents an individual experiment. *P < 0.05 by unpaired Student’s t-test; NS, not significant.
Our functional data are consistent with gain of function by the NLRC4 protein and support the mechanism of pathogenic macrophage activation with IL-1β and IL-18 overproduction and increased pyroptosis. Our data suggest for the first time, to our knowledge, that a macrophage-intrinsic defect can drive the MAS phenotype in the absence of a primary cytotoxic defect, thus providing a new paradigm for the pathogenesis of MAS. Activating mutations in NLRC4 cause a new autoinflammatory disease presenting as periodic fever syndrome with MAS, distinct from CAPS and Blau syndrome and partially responsive to IL-1 inhibition. The persistence of high serum IL-18 levels and macrophage hyper-responsiveness even after the initiation of IL-1–blocking treatment suggest a role for IL-18 in MAS and warrant an exploration of IL-18 as a therapeutic target.

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**ONLINE METHODS**

**Human subjects.** All research investigations were carried out as part of protocol 03-AR-0173 and, as such, were approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board. Written informed consent for the conduct of research and publication of findings was obtained from the parents of all affected individuals and healthy controls involved, and assent was obtained from the affected individuals and healthy controls when possible.

**Whole-exome sequencing.** The Agilent SureSelect Human 51Mb All Exon kit (Agilent Technologies) was used for exome capture. Sequencing was performed on Illumina HiSeq sequencers (Illumina) using 2 x 100 bp paired reads. Typical average on-target coverage was about 66x. A computational pipeline was developed to process the read data and perform tasks such as quality control and variant discovery, annotation and filtering. Briefly, the sequencing reads in FASTQ format were aligned to the human reference genome (GRCh-37) with the BWA (Burrows-Wheeler Aligner) mapping tool. The resulting BAM files (one per sample) were further processed to remove duplicate reads, refine alignment around indels and recalibrate base quality scores, according to the best-practice guideline for the Genome Analysis Toolkit (GATK) from the Broad Institute. A variant caller (UnifiedGenotyper) from GATK was used to make joint variant calls across multiple samples, and a variant quality score recalibration step was then performed by the GATK VQSR tool. In addition to being assigned quality scores, the variants were annotated by functional impact and allele frequency in public databases and local data sets. Sample relationship and sex were checked to identify potential sample mix-ups and false family relationships. Likely disease-causing mutations were selected and prioritized on the basis of quality score, allele frequency, functional impact, probable inheritance model (*de novo*, autosomal recessive, autosomal dominant or X linked) and expert evaluation.

**Sanger sequencing.** The reference sequence used for primer design and nucleotide numbering for NLRC4 was NM_001199139. The exonic regions and flanking intronic sites of the gene were amplified by PCR using specific primers (IDT) designed with Exon Primer. A list of all sequencing primers is included in Supplementary Table 3. The first PCR amplification reaction was performed in a final volume of 13 µl containing 10 picomoles of each primer, 6 µl of GoTaq Hot-Start Master Mix (Promega) and 20–200 ng of template DNA. Reactions were carried out for 35 cycles, with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. After PCR, the DNA fragments were treated with Exo-Sap (GE Healthcare Bioscience). PCR products underwent a second PCR with the ABI Prism BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and were directly sequenced using the ABI 3100 Genetic Analyzer (Applied Biosystems). Results were analyzed using DNAStar Lasergene software. Targeted sequencing of the other seven coding exons of NLRC4 was performed in the patient for the screening of SNPs.

**Gene expression analysis by RNA sequencing.** Total RNA was extracted from human blood samples collected in PAXGene tubes (PreAnalytix). Samples were obtained from five healthy pediatric controls, the patient with NLRC4-MAS at seven different time points, seven patients with NOMID with active disease before anakinra treatment and the same seven patients with NOMID with inactive disease after anakinra treatment. Complete blood counts were drawn on the same day for the patient with NLRC4-MAS. RNA integrity was analyzed with the Agilent 2100 Bioanalyzer. mRNA purification and fragmentation, cDNA synthesis and target amplification were performed using the Illumina TruSeq RNA Sample Preparation kit (Illumina). Poolcd cDNA libraries were sequenced using the Illumina HiSeq 2000 platform (Illumina). Sequencing results were analyzed using PartekGS v6.6 software and are expressed in RPKM (reads per kilobase of exon per million mapped). These results are available from the Gene Expression Omnibus (GEO) under accession GSE57253. All RPKM values were offset by the addition of 0.5 before analysis to limit the effects of samples with low expression levels.39 Lists of genes differentially expressed in the NLRC4-MAS flare sample and controls were generated without statistical analysis. Lists of genes differentially expressed in patients with NOMID and controls were generated by ANOVA analysis using Partek GS v6.6 software. Candidate gene lists for ‘inflammatory cytokines & receptors’, ‘apoptosis’, ‘inflammamasomes’ and ‘type I interferon response’ were obtained from the human RT’ Profiler, and a candidate gene list for ‘macrophage activation’ was derived from Biswas et al.40 (Supplementary Table 1). Genes overlapping between lists were eliminated. Differential expression of candidate genes was evaluated by determining the fold change between individual patient-derived samples and the average RPKM across healthy controls. When multiple transcript IDs were present for a gene, the ID with the highest average expression in healthy controls was chosen.

**Serum cytokine analysis.** Sera were stored at −80 °C. They were then analyzed using the Bio-Plex 21-plex and 27-plex Suspension Array systems in duplicate according to the manufacturer’s specifications. All sera were analyzed simultaneously to avoid batch effects. Values below the limit of detection were set to the limit of detection. Two-way hierarchical clustering was performed using Partek software.

**Plasmids.** A pCMV-SPORT6 vector containing the NLRC4 cDNA MGC:53530 was obtained from Thermo Scientific. Subsequent cloning was performed by Bioinnovative. Briefly, after DpnI digestion of the plasmid, the A–T mutation at position 1009 of NLRC4 was introduced using site-directed mutagenesis and complementary primers (Supplementary Table 3) containing the identified NLRC4 mutation. An IRES-EGFP sequence was then cloned downstream of the wild-type and mutant NLRC4 ORFs by seamless cloning. Finally, a 24-bp sequence encoding a C-terminal Flag tag was appended to each construct to generate pCMV-Sport6-NLRC4flag-GFP and pCMV-Sport6-T337SNLRC4flag-GFP. PCR fragments encoding either wild-type or Thr337Ser NLRC4-Flag were inserted into the multiple-cloning site of the migR1 vector41 by seamless cloning to generate NLRC4flag-MigR1 and T337SNLRC4flag-MigR1. All constructs were completely sequenced to confirm position.

**Transduction.** THP1 monocyes (American Type Culture Collection, TIB-202) were maintained at 100,000–600,000 cells/ml in RPMI 1640 containing 10% FCS, 50 µM β-mercaptoethanol, 50 U/ml streptomycin and 20 U/ml penicillin (R10 medium). Equal amounts of the empty MigR1, NLRC4flag-MigR1 and T337SNLRC4flag-MigR1 constructs were transfected into the Phoenix-Ampho cell line (American Type Culture Collection, CRL-3213) along with a pCl-Ampho helper plasmid. THP1 cells (1 x 10^6) were centrifuged with filtered, undiluted supernatants from the transfected Phoenix cells. Transduced THP1 cells were then grown as above in R10 medium supplemented with 1× Non-Essential Amino Acids (Gibco), 10 mM HEPES and 1 mM sodium pyruvate (R10C medium). Transduced cells were sorted for viability and GFP positivity on a BD FACSAria II about 1 week after transduction to >95% GFP positivity. Sorted cells were then passaged in R10C medium, and the stability of GFP expression was assessed by flow cytometry at least weekly. Two different transduced cell lines were generated in the same manner and were tested separately to ensure reproducibility. GFP positivity correlated with NLRC4 protein expression as assessed by immunoblotting (Fig. 5a). Cells were not routinely tested for contamination by mycoplasma.

**Antibodies and immunoblotting.** Transduced THP1 cells (5 x 10^5) were subjected to SDS-PAGE, transferred to nitrocellulose membranes and probed with appropriate antibodies. Antibodies used for protein blots were to NLRC4 (1:500 dilution; Millipore, 06-1125), procaspase-1 (1:1,000 dilution; Cell Signaling Technology, 2225), cleaved caspase-1 (1:500 dilution; Cell Signaling Technology, 4199) and HSPA8 (1:2,000 dilution; Cell Signaling Technology, 8444). Horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, 7074) and SuperSignal West Pico chemiluminescent substrate (Pierce) were used to detect primary antibodies. Equal protein loading was assessed by immunoblotting for HSPA8.

**Primary monocyte purification and generation of monocyte-derived macrophages.** Monocytes were isolated from peripheral blood mononuclear cells to >95% purity using CD14+ Magnetic Bead separation (Miltenyi) according to the manufacturer’s instructions. Monocytes were...
then stimulated directly. Monocyte-derived macrophages were generated by culturing purified monocytes in R10C medium supplemented with 10 ng/ml recombinant human M-CSF. Fresh culture medium was added every 2 d for 7 d.

**Generation of THP1 macrophages.** Stably transduced THP1 cells (1 × 10⁶) were cultured in R10C medium containing low-dose (10 ng/ml) PMA for 48 h to generate THP1-derived macrophages.

**Monocyte and macrophage stimulation.** After resting in medium, primary cells were primed with LPS (1 µg/ml) for 4 h before being stimulated with purified flagellin from *S. typhimurium* (FLA-ST, Invivogen) in DOTAP liposomes (IC-FLA; 6 µg DOTAP/1 µg FLA-ST; Roche) for an additional 2 h. IC-FLA stimulation was performed at 5 µg/ml unless otherwise indicated. For ATP stimulation, cells were primed in LPS for 5 h and then stimulated for 1 h with 1 mM ATP. LPS priming was omitted for time-course experiments.

**Supernatant cytokine measurement.** Comparison of cytokine production from primary human monocytes and monocyte-derived macrophages was performed in duplicate on undiluted culture supernatants using the Bio-Plex Xpress Suspension Array system, and results were read on a Bio-Rad Bio-Plex 200 HTF instrument according to the manufacturer’s instructions. IL-1β, IL-6, TNF-α, IL-10 (BD OptEIA) and IL-18 (MBL) levels from THP1 transduction experiments were measured by sandwich ELISA. For experiments comparing wild-type or mutant samples to empty vector samples, a nominal offset value near the limit of detection was added to each reading before normalization to minimize the effects of low values.

**Macrophage cell death.** Spontaneous and stimulated cell death was evaluated by measuring LDH release from 20,000 monocyte-derived macrophages using the Cytotox96 Non-Radioactive Cytotoxicity Assay according to the manufacturer’s instructions (Promega). No LPS priming was used for these experiments.

**Immunofluorescence and quantification of ASC aggregates.** Primary monocyte-derived macrophages were differentiated on glass coverslips as described above. Adherent macrophages were washed twice in PBS, primed with 10 ng/ml LPS for 6 h and stimulated with 5 µg/ml IC-FLA as noted. Cells were incubated with 2 µM biotinyl-YV AD-CMK (Enzo) during the last 4 h of stimulation. Cells were then rinsed with PBS, fixed and permeabilized with ice-cold methanol for 7 min at −20 °C, and blocked in PBS containing 1% fish skin gelatin (Sigma), 2% BSA and 5% normal goat serum for 1 h at room temperature. ASC was detected with rabbit antibody to ASC (Adipogen, AL177; 1:200 dilution) in blocking buffer (1 h at room temperature). A streptavidin-FITC conjugate (BD Biosciences) and Alexa Fluor 568–conjugated antibody to rabbit (Life Technologies) were used for secondary detection. Nuclei were counterstained with Hoechst 33342, and coverslips were mounted in Vectashield. Macrophages were analyzed on a Zeiss 780 confocal microscope using a 63× or 40× oil immersion objective. Quantification of ASC aggregates was performed visually at 63× magnification in at least 100 cells adjacent to an arbitrary line.

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