LETTERS

Cryopyrin activates the inflammasome in response to toxins and ATP

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A crucial part of the innate immune response is the assembly of the inflammasome, a cytosolic complex of proteins that activates caspase-1 to process the proinflammatory cytokines interleukin (IL)-1β and IL-18. The adaptor protein ASC is essential for inflammasome function[1,2], binding directly to caspase-1 (refs 3, 4), but the triggers of this interaction are less clear. ASC also interacts with the adaptor cryopyrin (also known as NALP3 or CIAS1)[5,6]. Activating mutations in cryopyrin are associated with familial cold autoinflammatory syndrome, Muckle–Wells syndrome and neonatal onset multisystem inflammatory disease, diseases that are characterized by excessive production of IL-1β[5,7]. Here we show that cryopyrin-deficient macrophages cannot activate caspase-1 in response to Toll-like receptor agonists plus ATP, the latter activating the $P2X_7$ receptor to decrease intracellular K^+ levels[8,9]. The release of IL-1β in response to nigericin, a potassium ionophore, and maitotoxin, a potent marine toxin, was also found to be dependent on cryopyrin. In contrast to *Asc*$^{-/-}$ macrophages, cells deficient in the gene encoding cryopyrin (*Cias1*$^{-/-}$) activated caspase-1 and secreted normal levels of IL-1β and IL-18 when infected with Gram-negative *Salmonella typhimurium* or *Francisella tularensis*. Macrophages exposed to Gram-positive *Staphylococcus aureus* or *Listeria monocytogenes*, however, required both ASC and cryopyrin to activate caspase-1 and secrete IL-1β. Therefore, cryopyrin is essential for inflammasome activation in response to signalling pathways triggered specifically by ATP, nigericin, maitotoxin, *S. aureus* or *L. monocytogenes*.

Cryopyrin-deficient mice (Supplementary Fig. S1) were generated by gene targeting to investigate the role of cryopyrin in inflammatory responses to pathogen-derived molecules. Cryopyrin-deficient (*Cias1*$^{-/-}$) macrophages stimulated with the Toll-like receptor-4 (TLR4) agonist lipopolysaccharide (LPS) phosphorylated IκBα and ERK normally (Fig. 1a), and they secreted normal amounts of TNF-α (Fig. 1b), IL-12 p40 (Fig. 1c), IL-6 and IL-10 (data not shown). Similar results were obtained using the TLR2 agonists Pam_3CSK_4 and heat-killed *L. monocytogenes* (HKLM) (data not shown). Our results show that cryopyrin is dispensable for NF-κB signalling by TLR2 and TLR4 in macrophages.

Because mutant variants of cryopyrin are associated with diseases in which IL-1β is produced in excess[5–7], we measured IL-1β released from *Cias1*$^{-/-}$ macrophages treated with TLR agonists and ATP (Fig. 1d). TLR agonists induce pro-IL-1β synthesis and ATP stimulates caspase-1-dependent cleavage and secretion of IL-1β[10]. In contrast to wild-type macrophages, which secreted readily detectable amounts of IL-1β and IL-18 in response to ATP plus ultra-pure LPS, Pam_3CSK_4, HKLM, R848 (TLR7/8 agonist), or CpG oligonucleotides (TLR9 agonist), *Cias1*$^{-/-}$ macrophages secreted negligible amounts of these cytokines (Fig. 1d, e). As shown previously[1,11], *Asc*$^{-/-}$ macrophages exhibited a similar defect in IL-1β and IL-18 production (Fig. 1d, e). Macrophages from heterozygous *Cias1*$^{+/-}$ mice secreted intermediate amounts of IL-1β and IL-18. C3H/HeJ macrophages expressing a non-functional form of TLR4 (ref. 12) secreted IL-1β and IL-18 in response to ATP plus either Pam_3CSK_4 or HKLM, but not LPS, demonstrating that our LPS was pure and not contaminated with other TLR agonists (Fig. 1d).

To determine whether IL-1β secretion from *Cias1*$^{-/-}$ macrophages was defective due to impaired pro-IL-1β synthesis and/or impaired caspase-1 activation, we immunoprecipitated [^{35}S]-methionine-labelled pro-IL-1β from LPS-primed macrophages. Wild-type, *Cias1*$^{+/-}$ and *Cias1*$^{-/-}$ macrophages produced comparable amounts of pro-IL-1β (Fig. 1f, left panel), indicating that defective IL-1β secretion from *Cias1*$^{-/-}$ cells was not due to impaired pro-IL-1β synthesis. Unlike their wild-type counterparts, however, *Cias1*$^{-/-}$ macrophages did not cleave pro-IL-1β after ATP treatment (Fig. 1f, right panel). This finding suggested that cryopyrin is essential for ATP-induced caspase-1 activation. A further indication of caspase-1 activation is its autocatalytic processing into p20 and p10 subunits. Western blotting for caspase-1 after LPS plus ATP treatment revealed the p10 and p20 subunits in wild-type but not *Cias1*$^{-/-}$ macrophages (Fig. 1g). Thus, cryopyrin is essential for activation of caspase-1 in response to LPS plus ATP. Notably, ATP was necessary but not sufficient for caspase-1 activation (Fig. 1g). TLR signalling is probably needed for expression of essential inflammasome components. For example, LPS stimulation of TLR4 increases expression of caspase-11, and analyses of caspase-11-deficient mice and cells demonstrate that caspase-11 is essential for inflammasome function[13].

To test whether the role of ATP in cryopyrin- and ASC-dependent caspase-1 activation relates to its ability to stimulate the $P2X_7$ receptor[9] and thereby reduce intracellular K^+ (ref. 8), we treated TLR-primed macrophages from wild-type, *Asc*$^{-/-}$ and *Cias1*$^{-/-}$ mice with nigericin or maitotoxin to deplete cytosolic K^+ (refs 14, 15). Wild-type macrophages primed with LPS or Pam_3CSK_4 secreted IL-1β and IL-18 in response to nigericin (Fig. 2a, b) or maitotoxin (Fig. 2c, d). By contrast, neither *Asc*$^{-/-}$ nor *Cias1*$^{-/-}$ macrophages released significant IL-1β or IL-18. Our data therefore show that ASC and cryopyrin are essential for IL-1β and IL-18 production by TLR-primed macrophages treated with agents that deplete intracellular K^+. Neither nigericin nor maitotoxin alone induced IL-1β release (Supplementary Fig. S2). Again, TLR signalling is probably required not only for the induction of pro-IL-1β (Fig. 1f) but also for expression of other proteins that are essential for inflammasome function.

To determine whether cryopyrin is essential for inflammation

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in vivo, wild-type and *Cias1*$^{-/-}$ mice were injected with a lethal dose of LPS to induce caspase-1-dependent endotoxic shock[16]. All of the wild-type mice died within 48 h, whereas only ~30% of the *Cias1*$^{-/-}$ mice had died after 72 h (Fig. 2e). Correlating with their enhanced survival, *Cias1*$^{-/-}$ mice had markedly less serum IL-1β and IL-18 than the wild-type mice (Fig. 2f). It is unclear why exogenous ATP is not required for IL-1β and IL-18 secretion *in vivo*. We speculate that other cell types impacted by LPS *in vivo* provide the ATP needed to engage the $P2X_7$ receptor. Our results demonstrate that cryopyrin, like ASC, is also an important mediator of LPS-induced endotoxic shock[1].

A previous study suggested that bacterial muramyl dipeptide (MDP) can activate a cryopyrin-containing inflammasome[17]. MDP is also the ligand for NOD2, which resembles cryopyrin in that it possesses a nucleotide binding domain and leucine-rich repeats[7,18–20]. Mutated NOD2 has been linked to Crohn's disease[7]. MDP induced phosphorylation of IκBα and ERK in wild-type and *Cias1*$^{-/-}$ macrophages but, in agreement with published results[19], *Nod2*$^{-/-}$ macrophages were unresponsive (Fig. 3a). NOD2-deficiency, however, did not impact on LPS-induced phosphorylation of IκBα and ERK, or subsequent IκBα degradation (Fig. 3a). In terms of cytokine production, MDP enhanced LPS-induced secretion of TNF and IL-12 p40 by wild-type and *Cias1*$^{-/-}$ macrophages but not *Nod2*$^{-/-}$ macrophages (Fig. 3b, c). Because cryopyrin was essential for LPS- plus ATP-induced IL-1β secretion, we could not assess whether MDP increased IL-1β production in wild-type cells by engaging cryopyrin (Fig. 3d). Our data indicate that cryopyrin is dispensable for MDP-induced activation of NF-κB and ERK, and confirm a crucial role for cryopyrin in ATP-induced activation of the macrophage inflammasome.

Next we determined whether cryopyrin is required for caspase-1 activation and IL-1β release when macrophages are infected by specific bacterial pathogens. Unlike ASC, cryopyrin was dispensable for normal caspase-1 activation, IL-1β secretion and macrophage

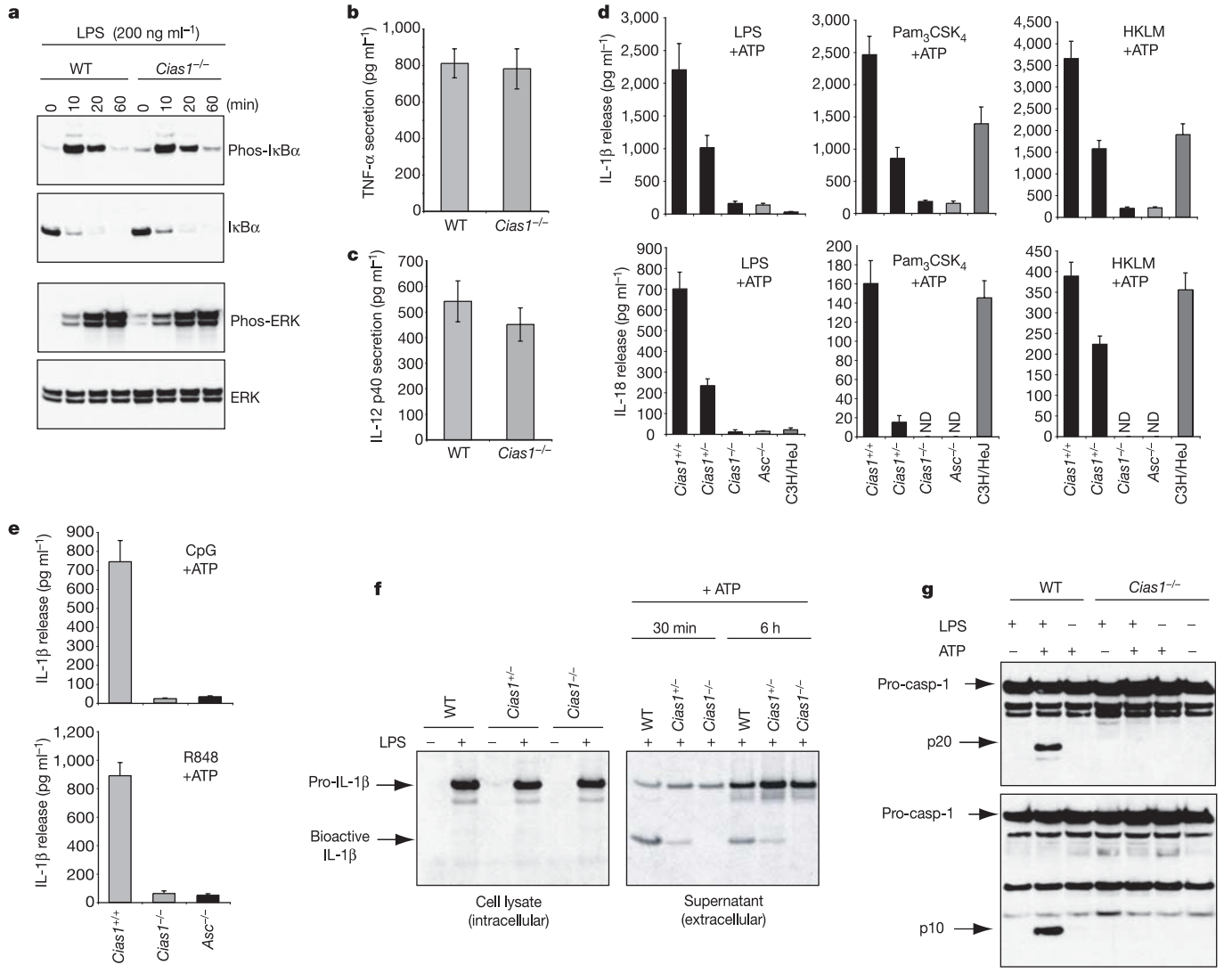

Figure 1 | Cryopyrin is essential for caspase-1 activation and IL-1β secretion in response to TLR agonists and ATP. **a**, Western blot analysis of phosphorylated and total IκBα and ERK in wild-type (WT) and *Cias1*$^{-/-}$ bone-marrow-derived macrophages stimulated with LPS. **b**, **c**, TNF (**b**) and IL-12 p40 (**c**) secretion by peritoneal macrophages cultured for 16 h with LPS. **d**, Macrophages primed for 16 h with LPS, Pam_3CSK_4, or HKLM were then pulsed with ATP. IL-1β or IL-18 released in the next 3 h is shown. ND, not detected. **e**, IL-1β secretion by macrophages primed with CpG DNA or R848 and then pulsed with ATP. Bars in **b**–**e** represent the mean ± s.d. of triplicate wells. Results are representative of four independent experiments. **f**, Immunoprecipitation of [^{35}S]-labelled pro-IL-1β and IL-1β from macrophages treated with LPS (left panel). Pro-IL-1β and IL-1β secreted after subsequent ATP treatment were immunoprecipitated from culture supernatants (right panel). **g**, Western blot analysis of caspase-1 in peritoneal macrophages stimulated with LPS for 16 h and then pulsed with ATP for 20 min. All *in vitro* experiments were performed with ultra-pure LPS.

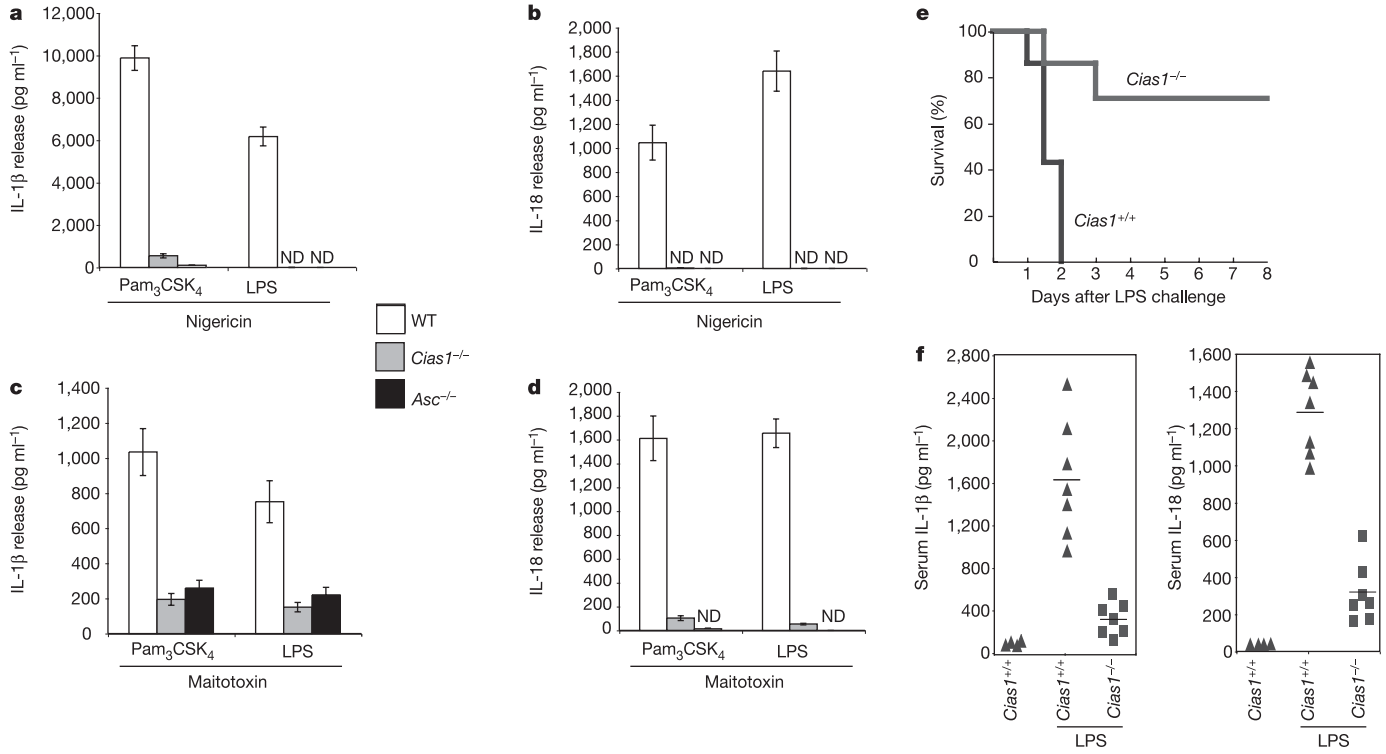

Figure 2 | Cryopyrin is essential for caspase-1 activation and IL-1β secretion in response to TLR agonists plus nigericin or maitotoxin. **a–d**, Wild-type, *Cias1*$^{-/-}$ and *Asc*$^{-/-}$ macrophages were primed with Pam_3CSK_4 or ultra-pure LPS and then treated with nigericin (**a**, **b**) or maitotoxin (**c**, **d**). IL-1β (**a**, **c**) and IL-18 (**b**, **d**) release was measured by ELISA. Bars in **a–d** represent the mean ± s.d. of triplicate wells. Results are representative of three independent experiments. ND, not detected. **e**, Survival of 8-week-old wild-type ($n = 7$) or *Cias1*$^{-/-}$ ($n = 7$) female mice injected intraperitoneally with crude LPS (40 mg kg^{-1} of body weight) on day 0. **f**, Levels of IL-1β and IL-18 in the serum of the mice in **e** at 3 h after injection. Lines indicate the mean serum level (IL-1β: WT, 1,581 pg ml^{-1}; *Cias1*$^{-/-}$, 304 pg ml^{-1}, $P = 0.001$; IL-18: WT, 1,321 pg ml^{-1}; *Cias1*$^{-/-}$, 329 pg ml^{-1}, $P = 0.0004$).

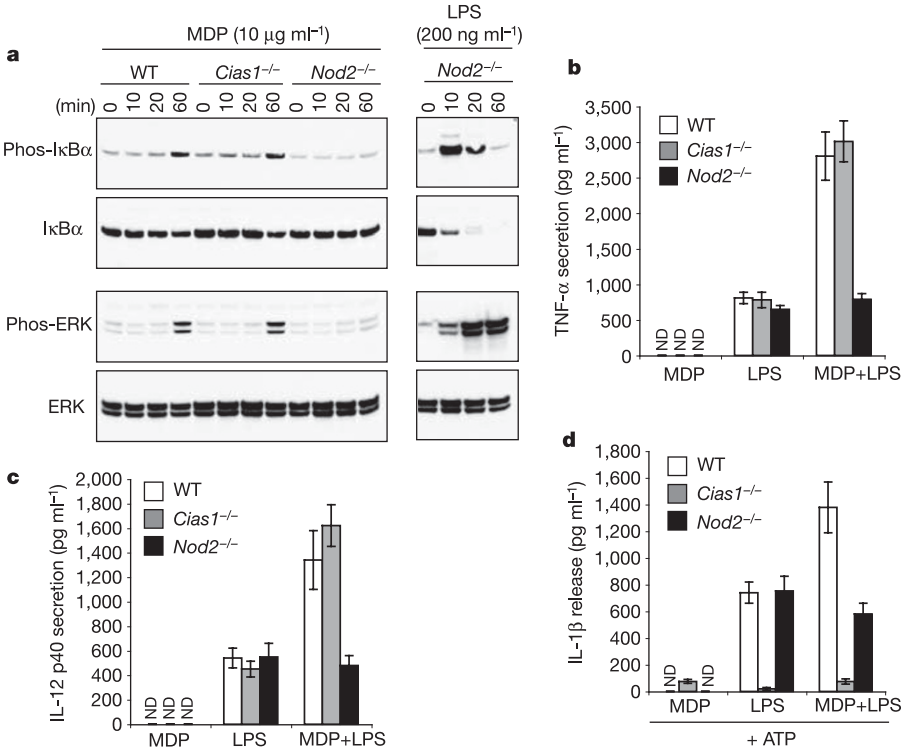

Figure 3 | MDP triggers NOD2-dependent, cryopyrin-independent NF-κB and ERK signalling. **a**, Western blot analysis of phosphorylated or total IκBα and ERK in wild-type, *Cias1*$^{-/-}$ and *Nod2*$^{-/-}$ macrophages stimulated with MDP or LPS. **b–d**, Secretion of TNF (**b**), IL-12 p40 (**c**), or IL-1β (**d**) by bone-marrow-derived macrophages treated with MDP and/or LPS. Macrophages in **d** were also pulsed with ATP. ND, not detected. Bars represent the mean ± s.d. of triplicate wells. Results are representative of three independent experiments.

cell death in response to Gram-negative *S. typhimurium* (Fig. 4a, b) or *F. tularensis* (Fig. 4c, d) (Supplementary Fig. S4). As shown previously[1,21], macrophages infected by *S. typhimurium* also require the adaptor protein Ipaf to activate caspase-1 (Fig. 4a). ASC and cryopyrin were essential for caspase-1 activation and secretion of IL-1β or IL-18 when macrophages were cultured with Gram-positive *S. aureus* (Fig. 4e–g) or *L. monocytogenes* (Fig. 4h–l), but NOD2 was dispensable (data not shown). ASC or cryopyrin deficiency caused a specific defect in IL-1β and IL-18 release because *Asc*$^{-/-}$ and *Cias1*$^{-/-}$ macrophages infected by *L. monocytogenes* and *S. aureus* yielded similar amounts of TNF to wild-type macrophages (Fig. 4k and data not shown). Macrophages cultured with *L. monocytogenes* deficient for the toxin listeriolysin O did not secrete IL-1β (Fig. 4l), so we speculate that listeriolysin O perturbs intracellular K^+ levels similar to extracellular ATP, nigericin and maitotoxin. Furthermore, live bacteria seem to be required because heat-killed *Listeria monocytogenes* (HKLM) induced very little IL-1β secretion (data not shown). S. *aureus* deficient in alpha-, beta- or gamma-toxin induced comparable IL-1β secretion to wild-type S. *aureus* (Supplementary Fig. S5), indicating that other *S. aureus* toxins may contribute to caspase-1 activation.

Our results suggest that the caspase-1 inflammasome is a dynamic entity that is assembled from different adaptor proteins in a stimulus-dependent manner (Fig. 4m). We show that cryopyrin is essential for inflammasome activation in response to signalling pathways triggered by specific bacterial infections and to treatments that deplete intracellular K^+. Ipaf and perhaps other members of the large NALP family of proteins might substitute for cryopyrin upon infection with Gram-negative bacteria such as *S. typhimurium* and *F. tularensis*. The cryopyrin-dependent response to extracellular ATP may represent a physiological response that occurs when ATP is released by dying cells and degranulating platelets. Future studies will need to address how activating mutations in cryopyrin circumvent the normal regulation of the inflammasome to produce inflammatory diseases such as familial cold autoinflammatory syndrome, Muckle–Wells syndrome and neonatal onset multisystem inflammatory disease[22–25]. An intriguing possibility is that low level bacterial infection coupled with a dysregulated

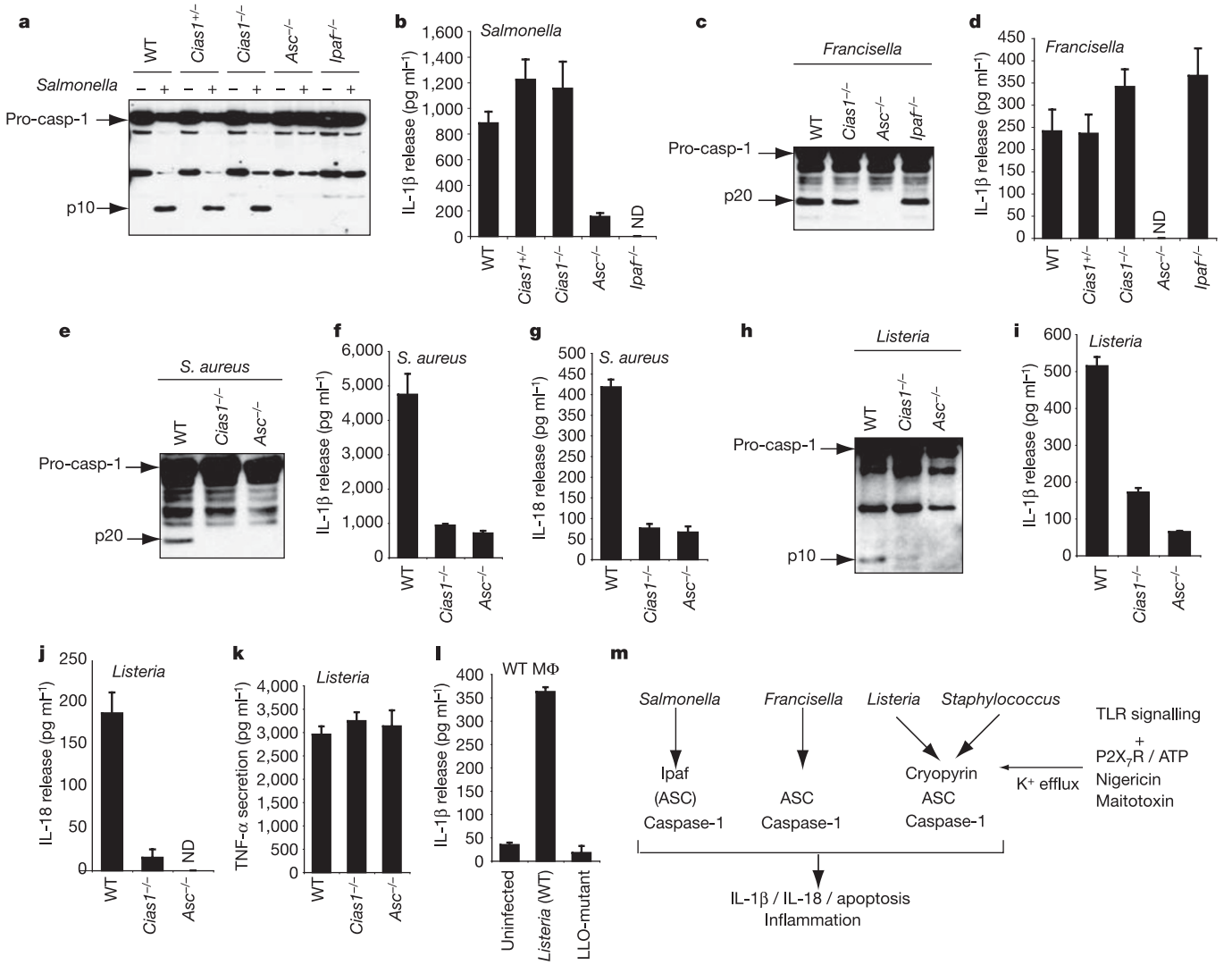

Figure 4 | *L. monocytogenes* and *S. aureus* induce cryopyrin-dependent caspase-1 activation and IL-1β secretion. **a**–**k**, Wild-type, *Cias1*$^{-/-}$, *Asc*$^{-/-}$ and *Ipaf*$^{-/-}$ macrophages pre-treated with LPS were infected with *S. typhimurium* (**a**, **b**), *F. tularensis* (**c**, **d**), *S. aureus* (**e**–**g**) or *L. monocytogenes* (**h**–**k**) and immunoblotted for the p10 or p20 subunit of caspase-1 (**a**, **c**, **e**, **h**). IL-1β (**b**, **d**, **f**, **i**), IL-18 (**g**, **j**) or TNF (**k**) secretion was measured by ELISA. ND, not detected. **l**, IL-1β released from wild-type macrophages pre-treated with LPS and infected with wild-type *L. monocytogenes* or a listeriolysin O (LLO) mutant. All data are representative of 2–4 independent experiments. Bars represent the mean ± s.d. of triplicate wells. LPS pre-treatment was not essential for IL-1β release (Supplementary Fig. 6). Cell viability after infection is shown in Supplementary Fig. 4. **m**, Model for the differential activation of the caspase-1 inflammasome by various pathogens and toxins.

cryopyrin response triggers the symptoms associated with these diseases.

METHODS

***Cias1*, *Nod2*, *Asc* and *Ipaf* mutant cells and mice.** *Asc*$^{-/-}$ and *Ipaf*$^{-/-}$ mice have been described[1]. *Cias1*$^{-/-}$ and *Nod2*$^{-/-}$ mice are described in Supplementary Figs 1 and 3. Macrophage and bacterial cultures are described in detail in the Supplementary Methods. Briefly, macrophages primed overnight with 50 ng ml^{-1} ultra-pure LPS were infected at a multiplicity of infection of 50 (30 for *F. tularensis*) for 1 h (*S. typhimurium*), 2.5 h (*L. monocytogenes*), 3 h (*S. aureus*) or 5 h (*F. tularensis*).

Immunoprecipitation, western blotting and pulse–chase analysis. IL-1β was immunoprecipitated with goat anti-mouse IL-1β (clone AF-401-NA; R&D Systems) and blotted with hamster anti-mouse IL-1β (clone B122; Becton Dickinson). For pulse–chase analyses, macrophages were treated with 1 μg ml^{-1} ultra-pure LPS for 3 h and then labelled with 200 μCi ml^{-1} [^{35}S]-methionine (ICN) for 45 min. Labelled cells were treated with 5 mM ATP (Sigma) for 30 min and then placed in fresh medium. Caspase-1 was blotted with rat anti-mouse caspase-1 (clone 4B4; Genentech) and rabbit anti-caspase-1 (sc-514; Santa Cruz Biotechnology). Phospho-IκBα (Ser32), IκBα, phospho-ERK and ERK antibodies were from Cell Signalling Technology.

Cytokine ELISAs. TNF and IL-12 p40 secretion were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems) after 16 h of stimulation with 500 ng ml^{-1} ultra-pure LPS (List Biologicals), 100 ng ml^{-1} Pam_3CSK_4 (Invivogen), 10^8 bacteria per ml HKLM (Invivogen), 5 μM CpG oligonucleotide (Invivogen ODN1826), 100 ng ml^{-1} R848 (Invivogen) or 10 μg ml^{-1} MDP (Invivogen). The macrophages then were pulsed for 20 min with 5 mM ATP, 20 μM nigericin (Calbiochem), or 0.5 nM maitotoxin (Dako) and cultured an additional 3 h for IL-1β and IL-18 secretion. Serum levels of IL-1β and IL-18 were determined 3 h after intraperitoneal injection with 40 mg kg^{-1} crude LPS (*Escherichia coli* serotype 0111:B4; Sigma).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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