Familial Mediterranean fever mutations lift the obligatory requirement for microtubules in Pyrin inflammasome activation

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Familial Mediterranean fever (FMF) is the most common monogenic autoinflammatory disease worldwide. It is caused by mutations in the inflammasome adaptor Pyrin, but how FMF mutations alter signaling in FMF patients is unknown. Herein, we establish *Clostridium difficile* and its enterotoxin A (TcdA) as Pyrin-activating agents and show that wild-type and FMF Pyrin are differentially controlled by microtubules. Diverse microtubule assembly inhibitors prevented Pyrin-mediated caspase-1 activation and secretion of IL-1 β and IL-18 from mouse macrophages and human peripheral blood mononuclear cells (PBMCs). Remarkably, Pyrin inflammasome activation persisted upon microtubule disassembly in PBMCs of FMF patients but not in cells of patients afflicted with other autoinflammatory diseases. We further demonstrate that microtubules control Pyrin activation downstream of Pyrin dephosphorylation and that FMF mutations enable microtubule-independent assembly of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) micrometer-sized perinuclear structures (specks). The discovery that Pyrin mutations remove the obligatory requirement for microtubules in inflammasome activation provides a conceptual framework for understanding FMF and enables immunological screening of FMF mutations.

FMF | Pyrin | inflammasome | colchicine | microtubules

Inflammasomes are multiprotein complexes that culminate in processing of caspase-1, thereby promoting maturation of proIL-1 β and proIL-18 into their active forms (1). Several inflammasome platforms have been described, and the concerted actions of inflammasomes frequently are of utmost importance for effective protection of the host against harmful environmental agents and infections (1). Conversely, mutations in genes coding for inflammasome components and regulators cause debilitating systemic autoinflammatory diseases, of which cryopyrin-associated periodic syndromes (CAPS; *NLRP3* mutations), autoinflammation with infantile enterocolitis (AIEC; *NLRP4* mutations), hyperimmunoglobulinemia syndrome (HIDS; *MVK* mutations) and familial Mediterranean fever (FMF; *MEFV* mutations) are notable examples (2, 3).

FMF is the most common monogenic autoinflammatory disease worldwide, affecting an estimated 150,000 patients (4). It typically has an autosomal recessive inheritance, and the clinical presentation is characterized by periodic fevers with childhood onset, frequently accompanied by serositis and joint pain (3, 4). The disease is highly prevalent in populations of the Eastern Mediterranean basin and the Middle East and has spread to the rest of the world with the extensive migrations of these populations (4, 5). More than 80% of FMF patients are homo- or

(compound) heterozygous for mutations in *MEFV*, the gene that codes for the inflammasome adaptor Pyrin (4, 5). More than 310 disease-associated variants in *MEFV* have been reported to date in the InFevr registry (6), with most residing in the C-terminal B30.2 (PRY/SPRY) domain of human Pyrin. Importantly, however, how FMF mutations regulate Pyrin signaling has remained enigmatic, and mouse studies of FMF are complicated by the absence of the B30.2 domain in murine Pyrin.

FMF alleles occur in as many as one of every four individuals of non-Ashkenazi Jew, Arab, Armenian, and Turkish descent (7–10). In addition, a subset of FMF patients is heterozygous for disease-associated *MEFV* alleles, and the clinical/functional relevance of some *MEFV* alleles is debated. Consequently, genetic analysis of FMF is sometimes inconclusive, and FMF diagnosis may be delayed for years (11). Although FMF is a systemic immunological disease, immunological diagnosis of the disease is currently not available and is likely to require further insight into how FMF mutations modulate Pyrin activation. The work presented

Significance

Familial Mediterranean fever (FMF) is an autoinflammatory disease caused by more than 310 mutations in the gene *MEFV*, which encodes Pyrin. Pyrin recently was shown to trigger inflammasome activation in response to Rho GTPase-modifying bacterial toxins. Here we report that *Clostridium difficile* infection and intoxication with its enterotoxin TcdA engage the Pyrin inflammasome. Moreover, activation of the Pyrin inflammasome, but not other inflammasomes, was hampered by microtubule-depolymerizing drugs in mouse and humans. Unexpectedly, we found that FMF mutations render Pyrin activation independent of microtubules. Thus, our findings provide a conceptual framework for understanding Pyrin signaling and enable functional diagnosis of FMF.

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Fig. 1. Microtubule depolymerizing drugs specifically inhibit the Pyrin inflammasome. (A and B) Wild-type LPS-primed BMDMs were pretreated with lumicolchicine or colchicine before stimulation with TcdA. Samples were immunoblotted for caspase-1 and IL-1 β (A), and supernatants were analyzed for IL-1 β (B). (C) Unprimed BMDMs were pretreated with colchicine before infection with *C. difficile* followed by supernatant collection and analysis of IL-1 β . (D–F) PBMCs from healthy donors ($n = 3$) were pretreated with lumicolchicine or colchicine before stimulation with TcdA. Samples were immunoblotted for IL-1 β (D), and supernatants were analyzed for IL-1 β (E) and IL-18 (F). (G–K) LPS-primed BMDMs (G and H) and PBMCs from healthy donors ($n = 3$) (I–K) were pretreated with colchicine, nocodazole, ABT-751, CA4P, or CYT997 before stimulation with TcdA. Samples were immunoblotted for caspase-1 and IL-1 β (G), and supernatants were analyzed for IL-1 β (H). PBMC samples were immunoblotted for IL-1 β (I), and supernatants were analyzed for IL-1 β (J) and IL-18 (K). (L–O) LPS-primed BMDMs were pretreated with colchicine before being stimulated with activators of the Nlrp1b (anthrax lethal toxin; LeTx) (L), Nlr4 (*S. Typhimurium*; STm) (M), AIM2 (dsDNA) (N), and Nlrp3 (nigericin; Nig) (O) inflammasomes followed by immunoblot to detect caspase-1 and IL-1 β . Black arrowheads indicate procaspase-1 and proIL-1 β , and white arrowheads indicate the p20 and p17 subunits. Luminex data are shown as mean $\pm$ SD, and all data are representative of at least three independent experiments.

colchicine abolished caspase-1 maturation as well as downstream cleavage and secretion of IL-1 β from TcdA-treated BMDMs (Fig. 1 A and B). Lumicolchicine, a structurally related colchicine photoisomer that does not bind tubulin (31), did not affect Pyrin activation (Fig. 1 A and B), demonstrating the specificity of these results. Colchicine also abolished Pyrin-mediated IL-1 β secretion from *C. difficile*-infected BMDMs (Fig. 1C). Consistent with these results, colchicine, but not lumicolchicine, inhibited Pyrin-induced proIL-1 β maturation in human PBMCs (Fig. 1D) and thus prevented the secretion of IL-1 β and IL-18 from these cells (Fig. 1 E and F). Importantly, a set of structurally unrelated microtubule polymerization inhibitors (nocodazole, ABT-751, CA4P, and

CYT997) also abolished the maturation of caspase-1, thereby hampering the ensuing cleavage and secretion of IL-1 β from TcdA-treated BMDMs (Fig. 1 G and H). Paralleling these results, these tubulin polymerization inhibitors prevented Pyrin-dependent IL-1 β maturation in human PBMCs (Fig. 1I). Similarly, secretion of IL-1 β and IL-18 from TcdA-treated PBMCs was significantly reduced (Fig. 1 J and K), establishing that microtubules are essential for human and murine Pyrin activation. These findings led us to examine the role of microtubule polymerization in other inflammasomes. Anthrax lethal toxin engaged Nlrp1b-dependent auto-maturation of caspase-1 and cleavage of proIL-1 β regardless of whether BMDMs had been pretreated with colchicine (Fig. 1L). Nlr4-driven caspase-1 activation and intracellular IL-1 β cleavage in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*)-infected macrophages also were normal in the presence of colchicine (Fig. 1M), as was activation of the AIM2 inflammasome by transfected dsDNA (Fig. 1N). Likewise, colchicine failed to modulate extracellular IL-1 β release by each of these inflammasomes (Fig. S6 A–C). Nigericin-induced Nlrp3 inflammasome activation in murine BMDMs and human PBMCs was insensitive to colchicine inhibition (Fig. 1O and Fig. S6 D–F), and nigericin-induced caspase-1 activation along with downstream maturation and release of IL-1 β continued unabated in the presence of the microtubule polymerization inhibitors nocodazole, ABT-751, CA4P, and CYT997 (Fig. S7). In conclusion, polymerized tubulin selectively and

Fig. 2. Differential microtubule regulation of Pyrin inflammasome activation identifies FMF patients. (A) PBMCs from healthy donors ($n = 7$) and FMF patients ($n = 2$) were infected with *C. difficile*, and the supernatant was analyzed for IL-1 β . (B) PBMCs from healthy donors ($n = 3$) and FMF patients ($n = 2$) were pretreated with Ac-YVAD-cmk before being stimulated with TcdA, and the supernatant was analyzed for IL-1 β . (C and D) PBMCs from healthy donors ($n = 7$) and FMF patients ($n = 2$) were stimulated with LPS for 5 h (C) or were pretreated with colchicine before stimulation with TcdA (D), and the supernatant was analyzed for IL-1 β . (E) PBMCs from healthy donors ($n = 3$) and FMF patients ($n = 2$) were pretreated with colchicine, nocodazole, ABT-751, CA4P, or CYT997 before stimulation with TcdA, and the supernatant was analyzed for IL-1 β . (F and G) PBMCs from healthy donors ($n = 5$) and from CAPS ($n = 4$), JIA ($n = 7$), and FMF ($n = 9$) patients were pretreated with colchicine before stimulation with TcdA, and the supernatant was analyzed for IL-1 β (F) and IL-18 (G). Luminex data are shown as mean $\pm$ SD, and all data are representative of at least three independent experiments. ns, non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Fig. 3. FMF-associated Pyrin binds tubulin but does not require microtubules for ASC speck assembly. (A) Schematic representation of different Flag-tagged Pyrin domain constructs. (B–D) Expression of different Flag-tagged Pyrin constructs in HEK293T cells followed by immunoprecipitation with Flag-beads and immunoblotting for tubulin and Flag. HEK293T cells were transfected with empty vector (EV), Flag-tagged wild-type (WT FL), or FMF (M694V) full-length human pyrin (FL) and its different domains, PYD, linker, PYD+linker and C-term, followed by immunoprecipitation with Flag-beads and immunoblotting for tubulin and Flag. (E and F) LPS-primed BMDMs (E) or HEK293T cells transfected with wild-type or FMF (M694V) full-length human pyrin (F) were pretreated with colchicine before stimulation with TcdA and then were immunoblotted to detect phosphorylated Pyrin (S241), IL-1 β , and β -actin (BMDMs), and phosphorylated Pyrin (S242), Flag, and tubulin (293T). PBMCs from healthy donors were pretreated with colchicine or CYT997 before stimulation with TcdA or nigericin (G and H). PBMCs from healthy donors and from CAPS, JIA, and FMF patients were pretreated with colchicine before stimulation with TcdA (I and J). ASC specks were analyzed by confocal microscopy as shown in micrographs (G and I) and automated quantification (H and J). Immunoblots and confocal images are representative of at least three independent experiments. ns, non-significant; *** P < 0.001; **** P < 0.0001.

critically controls activation of the Pyrin inflammasome in humans and rodents alike. Because murine Pyrin lacks the C-terminal B30.2 domain of human Pyrin, these results also imply that this region is dispensable for inflammasome activation and colchicine regulation. Nevertheless, the great majority of FMF mutations in human Pyrin localize to the C-terminal B30.2 domain (4).

Differential Microtubule Regulation of Pyrin Inflammasome Activation Identifies FMF Patients. We therefore sought to determine how mi-

croton tubules relate to Pyrin inflammasome signaling in FMF PBMCs. We reasoned that if microtubules relay an activating signal upstream of Pyrin, colchicine would halt TcdA-induced inflammasome activation in PBMCs of FMF patients. To test this hypothesis, we collected PBMCs from seven healthy controls and two FMF patients with confirmed *MEFV* mutations in the C-terminal B30.2 domain [Table S1, patients FMF1 (M694V/R761H) and FMF2 (M694I/M694I)]. We did not detect secreted IL-1 β in culture supernatants of untreated PBMCs of healthy donors or FMF patients (Fig. 2A). *C. difficile* infection triggered a substantial but comparable release of IL-1 β in wild-type and FMF PBMCs (Fig. 2A). FMF PBMCs that had been intoxicated with TcdA also secreted normal levels of IL-1 β , a response that was efficiently

blocked by the caspase-1 inhibitor Ac-YVAD-cmk (Fig. 2B). Moreover, IL-1 β levels secreted by FMF PBMCs in response to the NLRP3 inflammasome stimulus LPS (32, 33) were comparable to those of healthy donors (Fig. 2C). These results indicate that FMF mutations are not hypermorphic for inflammasome activation relayed by either Pyrin or NLRP3. Moreover, they suggest that FMF mutations differ from CAPS-linked mutations in *NLRP3* that significantly enhance LPS- and cold-induced Nlrp3 inflammasome activation (33, 34). Remarkably, however, although colchicine pretreatment abolished TcdA-induced IL-1 β secretion from PBMCs of healthy individuals (Fig. 2D), it augmented the TcdA-induced IL-1 β secretion from FMF PBMCs (Fig. 2D). The microtubule assembly inhibitors nocodazole, ABT-751, CA4P, and CYT997 also had opposite influences on TcdA-induced IL-1 β secretion from FMF PBMCs and from PBMCs of healthy donors (Fig. 2E). Furthermore, IL-18 release from TcdA-treated wild-type PBMCs was blunted by colchicine and other microtubule polymerization inhibitors, whereas FMF PBMCs resisted suppression (Fig. S8). To validate these results further and to test whether resistance to colchicine inhibition was a defining feature of FMF PBMCs, we repeated our studies with PBMCs from a larger group of healthy donors ($n = 5$) and from an additional cohort of FMF

patients with a variety of defined *MEFV* mutations ($n = 9$; Table S1). PBMCs from patients afflicted with CAPS disease resulting from heterozygous mutations in *NLRP3* ($n = 4$; Table S1) and from patients diagnosed with juvenile idiopathic arthritis (JIA, systemic and nonsystemic; $n = 7$) were tested also. As with PBMCs from healthy donors, colchicine blocked TcdA-induced IL-1 β and IL-18 secretion from PBMCs of CAPS and JIA patients, indicating that their Pyrin inflammasome responses were regulated identically to those of healthy individuals (Fig. 2 *F* and *G*). In marked contrast, however, all FMF patients continued to secrete significant IL-1 β and IL-18 levels after colchicine pretreatment (Fig. 2 *F* and *G*). We verified that colchicine efficiently disrupted assembled microtubules in the PBMCs of healthy individuals and FMF patients alike (Fig. S9), ruling out the remote possibility that microtubules of FMF PBMCs resisted microtubule disassembly by colchicine. Together, these results suggest that FMF mutations converge on lifting the critical requirement for microtubules in Pyrin inflammasome activation.

FMF-Associated Pyrin Binds Tubulin but Does Not Require Microtubules for ASC Speck Assembly. Dephosphorylation of Pyrin's intermediate linker domain was recently shown to be required for TcdA- and TcdB-induced inflammasome activation (14, 15, 19), and introduction of FMF mutations in the B30.2 domain of ectopically expressed Pyrin did not interfere with this process (15). In agreement with these reports, we showed that endogenous Pyrin was not constitutively active in FMF PBMCs but was engaged only after TcdA stimulation or *C. difficile* infection (Fig. 2 and Fig. S8). To characterize further how FMF mutations alter Pyrin-dependent inflammasome activation, we examined the binding of ectopically expressed Pyrin domains to endogenous tubulin (Fig. 3*A*). Flag-fused constructs corresponding respectively to the N-terminal PYD domain, the intermediate linker sequence, the PYD+linker combination, or the C-terminal tripartite motif (TRIM) and B30.2 domains were expressed in 293T cells and were immunoprecipitated with Flag-beads. Constructs containing either the PYD or the C-terminal regions of wild-type Pyrin coprecipitated endogenous tubulin, but the intermediate linker sequence did not (Fig. 3*B*). This result suggests that Pyrin interacts with tubulin through both its N and C termini. However, introduction of the FMF-associated M694V mutation did not prevent tubulin binding in either full-length Pyrin (Fig. 3*C*) or in the isolated carboxyl-terminal region (Fig. 3*D*). As reported (19), TcdA also triggered Pyrin dephosphorylation in wild-type BMDMs that had been pretreated with colchicine (Fig. 3*E*). Furthermore, colchicine failed to prevent TcdA-induced dephosphorylation of ectopically expressed wild-type Pyrin and the FMF-associated M694V Pyrin mutant in 293T cells (Fig. 3*F*). In agreement with our other studies showing that microtubule polymerization inhibitors prevented inflammasome activation selectively in wild-type, but not in FMF, PBMCs, this finding positions microtubules in the pathway downstream of Pyrin dephosphorylation.

PYD-based inflammasome sensors such as Pyrin nucleate ASC filaments through a biphasic mechanism in which their PYD domain first nucleates ASC PYD to oligomerize into prion-like fibers that then condense into ASC specks through ASC–caspase recruitment domain (CARD) interactions that also enable the recruitment of caspase-1 zymogens (35). Interference with these first and second stages of ASC speck assembly therefore results in either the complete absence or the formation of atypical filamentous fibers, respectively (35). We exposed PBMCs of healthy donors to TcdA or nigericin to trigger ASC speck assembly through the Pyrin and NLRP3 inflammasomes, respectively (Fig. 3 *G* and *H*). Colchicine and the unrelated microtubule polymerization inhibitor CYT997 selectively prevented ASC speck formation in response to TcdA but not in nigericin-treated PBMCs (Fig. 3 *G* and *H*), demonstrating that microtubules were selectively required for nucleation of ASC specks by Pyrin but not by NLRP3. As in healthy donors, colchicine abolished TcdA-induced ASC speck assembly in PBMCs of CAPS and JIA patients (Fig. 3 *I* and *J*). In marked contrast, however, the assembly of ASC specks by FMF PBMCs was unabated in the presence of colchicine (Fig. 3 *I* and *J*). Similar results were obtained with the unrelated microtubule

polymerization inhibitor CYT997 (Fig. S10). Thus, FMF mutations in Pyrin remove the critical dependency on microtubules for ASC speck assembly and inflammasome activation downstream of Pyrin dephosphorylation.

Discussion

The observation that Pyrin inflammasome activation by TcdA, TcdB, and live *C. difficile* infection required intact microtubules in both human PBMCs and murine macrophages implies that the C-terminal B30.2 domain, which harbors most FMF mutations in humans but is absent in mouse Pyrin, is dispensable for inflammasome activation. Although the B30.2 domain is dispensable for Pyrin inflammasome activation, we established here that FMF mutations in this domain nonetheless remove the critical reliance on intact microtubules for Pyrin-based nucleation of ASC specks and inflammasome signaling. Microtubules were recently proposed to control inflammasome activation apically of Pyrin dephosphorylation in response to bacterial RhoA inactivation (14). However, this suggestion is difficult to reconcile with the observation that TcdA-induced Pyrin dephosphorylation continued unhampered in colchicine-pretreated macrophages and 293T cells (Fig. 3 *E* and *F* and ref. 19). Moreover, we showed that FMF mutations render Pyrin activation independent of microtubules. Thus, our results provide a conceptual framework for understanding FMF based on a mechanistic model of Pyrin signaling in which microtubules control inflammasome activation downstream of Pyrin dephosphorylation (Fig. 4). In this model, microtubules relay an activating signal to dephosphorylated wild-type Pyrin that shifts autorepressed Pyrin into an open conformation. FMF mutations in the human B30.2 domain may force dephosphorylated Pyrin in an open conformation that readily binds the inflammasome adaptor ASC, effectively replacing microtubule-relayed signals (Fig. 4).

Paradoxically, although we show here that FMF mutations render Pyrin inflammasome activation insensitive to colchicine, this drug is an effective treatment that suppresses periodic inflammatory attacks in the majority of FMF patients and prevents amyloidosis, a major long-term complication of the disease that may result in renal failure and death (5, 36). However, the clinical efficacy of colchicine treatment is likely associated with its ability to decrease leukocyte motility and phagocytosis during inflammation (37, 38). Recent studies suggested that defects in the mevalonate pathway seen in the hereditary autoinflammatory disease mevalonate kinase deficiency (MKD), also named “hyperimmunoglobulinemia D syndrome” (HIDS), may also trigger unwarranted activation of the Pyrin inflammasome (14, 39).

Fig. 4. Schematic model of Pyrin inflammasome activation by RhoA-modifying toxins. *C. difficile* TcdA and TcdB inactivate RhoA, thereby triggering dephosphorylation of Pyrin and its release from inhibitory 14-3-3 proteins. Microtubules are critical for activation of wild-type Pyrin in human PBMCs and murine macrophages. In contrast, FMF-associated mutations in Pyrin render ASC speck assembly and inflammasome activation independent of microtubules.

In contrast to FMF patients, however, MKD patients generally do not benefit from colchicine treatment, whereas blockade of IL-1 has shown promising results (40). Given our observations that FMF mutations render Pylrin activation resistant to colchicine blockade, it would be interesting to investigate the role of microtubules in MKD-associated inflammasome activation.

Akin to colchicine, certain pathogens express toxins that manipulate microtubule dynamics, as exemplified by the CDT toxin of hypervirulent *C. difficile* strains (41). It therefore is tempting to speculate that the high frequency of heterozygous *MEFV* mutations in endemic FMF regions (42) might be related to their rendering Pylrin activation insensitive to microtubule manipulations by such pathogens. Given the key role of inflammasomes in antimicrobial host defense (43), the ability to engage the Pylrin inflammasome in the presence of microtubule dynamics blockade is likely to have offered heterozygous individuals a selective advantage in clearing such infections. Finally, the insight that inflammasome activation by FMF Pylrin resists colchicine blockade enables functional/immunological screening of the disease among clinically overlapping autoinflammatory patients and thus may contribute to timely diagnosis and commencement of therapy in the future.

1. Van Gorp H, Kuchmiy A, Van Hauwermeiren F, Lamkanfi M (2014) NOD-like receptors interfacing the immune and reproductive systems. *FEBS J* 281(20):4568–4582.
2. McGonagle D, McDermott MF (2006) A proposed classification of the immunological diseases. *PLoS Med* 3(8):e297.
3. Masters SL, Simon A, Aksentijevich I, Kastner DL (2009) Hereditary autoinflammatory diseases: The molecular pathophysiology of autoinflammatory disease. *Annu Rev Immunol* 27: 621–668.
4. Portincasa P (2016) Colchicine, biologic agents and more for the treatment of familial Mediterranean fever. The old, the new, and the rare. *Curr Med Chem* 23(1):60–86.
5. Ozen S, Bilginer Y (2014) A clinical guide to autoinflammatory diseases: Familial Mediterranean fever and next-of-kin. *Nat Rev Rheumatol* 10(3):135–147.
6. Milhavet F, et al. (2008) The infevers autoinflammatory mutation online registry: Update with new genes and functions. *Hum Mutat* 29(6):803–808.
7. French FMF Consortium (1997) A candidate gene for familial Mediterranean fever. *Nat Genet* 17(1):25–31.
8. The International FMF Consortium (1997) Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. *Cell* 90(4): 797–807.
9. Daniels M, Shohat T, Brenner-Ullman A, Shohat M (1995) Familial Mediterranean fever: High gene frequency among the non-Ashkenazic and Ashkenazic Jewish populations in Israel. *Am J Med Genet* 55(3):311–314.
10. Rogers DB, et al. (1989) Familial Mediterranean fever in Armenians: Autosomal recessive inheritance with high gene frequency. *Am J Med Genet* 34(2):168–172.
11. Toplak N, et al.; Paediatric Rheumatology International Trials Organisation (PRINTO), Eurotraps and Eurofever Projects (2012) An international registry on auto-inflammatory diseases: The Eurofever experience. *Ann Rheum Dis* 71(7):1177–1182.
12. Gavrilin MA, et al. (2012) Activation of the pyrin inflammasome by intracellular Burkholderia cenocepacia. *J Immunol* 188(7):3469–3477.
13. Xu H, et al. (2014) Innate immune sensing of bacterial modifications of Rho GTPases by the Pylrin inflammasome. *Nature* 513(7517):237–241.
14. Park YH, Wood G, Kastner DL, Chae JJ (2016) Pylrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. *Nat Immunol* 17(8): 914–921.
15. Masters SL, et al. (2016) Familial autoinflammation with neutrophilic dermatitis reveals a regulatory mechanism of pyrin activation. *Sci Transl Med* 8(332):332ra45.
16. Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ (2016) Clostridium difficile infection. *Nat Rev Dis Primers* 2:16020.
17. Coll RC, et al. (2015) A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med* 21(3):248–255.
18. Perregaux DG, et al. (2001) Identification and characterization of a novel class of interleukin-1 post-translational processing inhibitors. *J Pharmacol Exp Ther* 299(1): 187–197.
19. Gao W, Yang J, Liu W, Wang Y, Shao F (2016) Site-specific phosphorylation and microtubule dynamics control Pylrin inflammasome activation. *Proc Natl Acad Sci USA* 113(33):E4857–E4866.
20. Hasegawa M, et al. (2011) Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen. *J Immunol* 186(8):4872–4880.
21. Wu J, Fernandes-Alnemri T, Alnemri ES (2010) Involvement of the AIM2, NLR4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. *J Clin Immunol* 30(5):693–702.
22. Rathinam VA, et al. (2010) The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11(5):395–402.

Materials and Methods

All reported patients and healthy controls provided written informed consent for participation in the study, in accordance with International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use/Good Clinical Practice (ICH/GCP) guidelines. The research protocol was approved by the ethics committee of Ghent University Hospital under number 2012_593 and the protocols of Bambino Gesù Children's Hospital.

All mice were kept in specific pathogen-free conditions within the animal facilities of Ghent University. All animal experiments were approved by the ethics committee on laboratory animal welfare of Ghent University.

Detailed methods used in all experiments throughout this work are described in *SI Materials and Methods*.

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23. Bauernfeind FG, et al. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183(2):787–791.
24. Van Oudenbosch N, et al. (2014) Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1 autoproteolysis and speck formation. *Nat Commun* 5:3209.
25. Carlson BA, et al. (2009) Selenoproteins regulate macrophage invasiveness and extracellular matrix-related gene expression. *BMC Immunol* 10:57.
26. Chen X, et al. (2012) Requirement for the histone deacetylase Hdac3 for the inflammatory gene expression program in macrophages. *Proc Natl Acad Sci USA* 109(42): E2865–E2874.
27. Litvak V, et al. (2009) Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* 10(4):437–443.
28. Rodgers MA, et al. (2014) The linear ubiquitin assembly complex (LUBAC) is essential for NLRP3 inflammasome activation. *J Exp Med* 211(7):1333–1347.
29. Rowley SM, et al. (2014) Tumor progression locus 2 (Tpl2) kinase promotes chemokine receptor expression and macrophage migration during acute inflammation. *J Biol Chem* 289(22):15788–15797.
30. Mansfield E, et al. (2001) The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments. *Blood* 98(3):851–859.
31. Bryan J (1972) Definition of three classes of binding sites in isolated microtubule crystals. *Biochemistry* 11(14):2611–2616.
32. Netea MG, et al. (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 113(10):2324–2335.
33. Lamkanfi M, et al. (2009) Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J Cell Biol* 187(1):61–70.
34. Hoffman HM, et al. (2004) Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 364(9447):1779–1785.
35. Lu A, et al. (2014) Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell* 156(6):1193–1206.
36. Giancane G, et al. (2015) Evidence-based recommendations for genetic diagnosis of familial Mediterranean fever. *Ann Rheum Dis* 74(4):635–641.
37. Goldfinger SE (1972) Colchicine for familial Mediterranean fever. *N Engl J Med* 287(25):1302.
38. Lidar M, et al. (2004) Colchicine nonresponsiveness in familial Mediterranean fever: Clinical, genetic, pharmacokinetic, and socioeconomic characterization. *Semin Arthritis Rheum* 33(4):273–282.
39. Akula MK, et al. (2016) Control of the innate immune response by the mevalonate pathway. *Nat Immunol* 17(8):922–929.
40. Ter Haar N, et al.; Paediatric Rheumatology International Trials Organisation (PRINTO) and the Eurofever/Eurotraps Projects (2013) Treatment of autoinflammatory diseases: Results from the Eurofever Registry and a literature review. *Ann Rheum Dis* 72(5):678–685.
41. Schwan C, et al. (2009) Clostridium difficile toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog* 5(10): e1000626.
42. Schaner P, et al. (2001) Episodic evolution of pyrin in primates: Human mutations recapitulate ancestral amino acid states. *Nat Genet* 27(3):318–321.
43. Lamkanfi M, Dixit VM (2011) Modulation of inflammasome pathways by bacterial and viral pathogens. *J Immunol* 187(2):597–602.
44. Kayagaki N, et al. (2011) Non-canonical inflammasome activation targets caspase-11. *Nature* 479(7371):117–121.
45. Vande Walle L, et al. (2014) Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. *Nature* 512(7512):69–73.