

## Original article

## Familial Mediterranean fever mutations are hypermorphic mutations that specifically decrease the activation threshold of the Pyrin inflammasome

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## Abstract

**Objectives.** FMF is the most frequent autoinflammatory disease and is associated in most patients with bi-allelic *MEFV* mutations. *MEFV* encodes Pyrin, an inflammasome sensor activated following RhoGTPase inhibition. The functional consequences of *MEFV* mutations on the ability of Pyrin variants to act as inflammasome sensors are largely unknown. The aim of this study was to assess whether *MEFV* mutations affect the ability of Pyrin to detect RhoGTPase inhibition and other inflammasome stimuli.

**Methods.** IL-1 $\beta$  and IL-18 released by monocytes from healthy donors (HDs) and FMF patients were measured upon specific engagement of the Pyrin, NLRP3 and NLRC4 inflammasomes. Cell death kinetics following Pyrin activation was monitored in real time.

**Results.** Monocytes from FMF patients secreted significantly more IL-1 $\beta$  and IL-18 and died significantly faster than HD monocytes in response to low concentrations of *Clostridium difficile* toxin B (TcdB), a Pyrin-activating stimulus. Monocytes from patients bearing two *MEFV* exon 10 pathogenic variants displayed an increased Pyrin inflammasome response compared with monocytes from patients with a single exon 10 pathogenic variant indicating a gene-dosage effect. Using a short priming step, the response of monocytes from FMF patients to NLRP3- and NLRC4-activating stimuli was normal indicating that *MEFV* mutations trigger a specific hypersensitivity of monocytes to low doses of a Pyrin-engaging stimulus.

**Conclusion.** Contrary to the *NLRP3* mutations described in cryopyrin-associated periodic syndrome, FMF-associated *MEFV* mutations do not lead to a constitutive activation of Pyrin. Rather, FMF-associated mutations are hypermorphic mutations that specifically decrease the activation threshold of the Pyrin inflammasome without affecting other canonical inflammasomes.

**Key words:** familial mediterranean fever, *MEFV*, inflammasome, Pyrin, NLRP3, NLRC4, autoinflammation, IL-1 $\beta$ , IL-18

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## Rheumatology key messages

- FMF-associated Pyrin variants detect RhoGTPase inhibition confirming that *MEFV* mutations are not loss-of-function mutations.
- *MEFV* mutations induce a decreased threshold of activation of the Pyrin inflammasome in FMF.
- The Pyrin inflammasome response in FMF is dependent on a gene-dosage effect.

## Introduction

FMF is the most common hereditary autoinflammatory disorder. It is characterized by recurrent episodes of fever, polyserositis and abdominal pain [1]. The main long-term complication of FMF is renal amyloidosis [2]. Treatment with colchicine prevents disease flares and amyloidosis. FMF is most often inherited in a recessive manner [3, 4]. Accordingly, the majority of FMF patients hold bi-allelic pathogenic variants in *MEFV*, a gene encoding Pyrin, an inflammasome sensor. While numerous *MEFV* sequence variants have been reported (314 in the Infevers registry [5]), the impact of these variants on Pyrin functions remains unknown. The sequence variants in exon 10, which include the most prevalent mutation, p.M694V, have been demonstrated in genotype–phenotype correlation studies to be the most pathogenic variants [6, 7]. Yet, a number of FMF patients display a single clearly pathogenic *MEFV* variant [8] highlighting the complexity of the disease and the need for a better understanding of the underlying mechanisms.

Pyrin is an inflammasome sensor that detects an imbalance in RhoGTPases activity, such as the one caused by bacterial effectors or bacterial toxins [9–11]. The toxin B from *Clostridium difficile* (TcdB) is a paradigm of such Pyrin-activating virulence factors [9]. TcdB is a glycosylating enzyme that inactivates the RhoA GTPase, which results in activation of the Pyrin inflammasome [9]. Inflammasomes are immune platforms leading to caspase-1 activation, release of the proinflammatory cytokines IL-1 $\beta$  and IL-18 and to an inflammatory cell death termed pyroptosis [12]. Different sensors define the specificity of the inflammasomes. Furthermore, while the canonical inflammasomes rely on caspase-1 as their sole enzymatic effector, alternative and non-canonical inflammasomes implicating caspases-4/5 and 8 have been described [13, 14]. The inflammasomes are key immune complexes for resisting microbial infections [15], yet mutations in inflammasome sensor-encoding genes are responsible for several hereditary autoinflammatory syndromes. *NLRP3* mutations cause the autosomal-dominant cryopyrin-associated periodic syndrome (CAPS) [16]. Gain-of-function mutations in *NLR4* trigger severe inflammation and (NLR4-macrophage activation syndromes) [17]. Similarly, a specific gain-of-function mutation in *MEFV* (p.S242R) causes pyrin-associated autoinflammation with neutrophilic dermatosis, an entity distinct from FMF [18].

How *MEFV* mutations in FMF patients alter the activity of inflammasomes is still unresolved. At odds with the recessive inheritance, functional data obtained in mouse models lacking *MEFV* or expressing various human *MEFV* variants indicate that FMF mutations are gain-of-function mutations [19]. Accordingly, monocytes from

FMF patients release more IL-1 $\beta$  than monocytes from healthy donors (HDs) upon long-term exposure (18 h) to lipopolysaccharide (LPS) [20]. A controversy exists as to whether the FMF phenotype is NLRP3-independent (as demonstrated in mouse models of FMF [19]) or -dependent as demonstrated using primary human monocytes [20]. Furthermore, the specific impact of *MEFV* sequence variations on the Pyrin inflammasome response remains largely unexplored although a very recent paper suggested that the Pyrin inflammasomes of FMF patients and HDs were equally able to detect bacterial toxin-mediated RhoGTPase modifications [21].

Here, using a matrix of inflammasome stimuli and several inflammasome readouts, we demonstrate that, using a short priming step, monocytes from FMF patients respond stronger and faster to low doses of TcdB than monocytes from HDs. This result indicates that *MEFV* mutations are hypermorphic mutations, that is, that the encoded Pyrin variants do not gain a novel stimulus-independent function but have an increased ability to sense toxin-mediated RhoGTPase inhibition. Furthermore, we demonstrate that the number of exon 10 pathogenic variants impacts the strength of the Pyrin inflammasome response demonstrating a gene-dosage effect, which correlates with the previously described gene-dosage effects on the levels of IL-1 $\beta$  released following long term LPS treatment [20] and on the severity of the FMF clinical symptoms [22]. The higher reactivity of the Pyrin inflammasome toward its specific stimulus in FMF patients compared with HD is likely to contribute to the inflammatory flares.

## Methods

Supplementary methods are available, with sections Subjects and Cytokine detection and cell death assay, at *Rheumatology* online.

## Subjects

Forty-eight patients with FMF were included along with 3 with CAPS and 26 HDs (supplementary Table S1, available at *Rheumatology* Online). All FMF patients fulfilled the Tel Hashomer criteria for FMF and had at least one mutation in the *MEFV* gene. Blood samples from HDs were drawn on the same day as patients.

## Ethics statement

The study was approved by the French Comité de Protection des Personnes (CPP, no. L16-189) and by the French Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé (CCTIRS, no. 16.864). The authors observed a

strict accordance to the guidelines of the Declaration of Helsinki and informed written consent was obtained from every patient or their legal representative. HD blood was provided by the Etablissement Français du Sang in the framework of the convention no. 14-1820.

### Monocyte isolation

Blood was drawn in heparin-coated tubes and kept at room temperature overnight. The next day, peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Lymphocyte Separation Medium (Eurobio, Courtaboeuf, France) [23]. Monocytes were isolated from PBMCs by magnetic selection using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) [24] and the AutoMACS Pro Separator (Miltenyi Biotec) following the manufacturer's instructions. Monocytes were enumerated in the presence of a viability marker [propidium iodide (PI), 10 µg/ml] by flow cytometry (BD Accuri C6 Flow Cytometer, BD, Bury Saint Edmunds, UK) [25].

### Inflammasome activation

Monocytes were seeded in 96-well plates (unless otherwise indicated at  $5 \times 10^3$  cells/well) in RPMI 1640, GlutaMAX medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (Lonza, Bâle, Switzerland). The selected cell number was defined in preliminary experiments as the minimal cell number allowing a robust IL-1 $\beta$  secretion in the presence of LPS + signal 2 (supplementary Fig. S1A, available at *Rheumatology* Online). Monocytes were incubated for 3 h in the presence or absence of a priming signal consisting of LPS (10 ng/ml, Invivogen, San Diego, CA, USA) or muramyl dipeptide (MDP; 100 ng/ml, Invivogen) [14, 26]. Unless otherwise indicated, cells were then treated for 1.5 h with inflammasome activators as follows: for NLRP3 inflammasome: nigericin (5 µM, Invivogen) and adenosine triphosphate (ATP; 2.5 mM, Sigma-Aldrich, Saint-Louis, MO, USA) [27]; for NLRC4 inflammasome: lethal factor N-terminal domain (LFn) of *Bacillus anthracis* fused to the PrgI protein of the type III secretion system of *Salmonella enterica* serovar *Typhimurium* (LFn-PrgI; 50 ng/ml [28], produced at the Institute of Biology and Chemistry of Proteins (IBCP), Lyon, France; the corresponding plasmid was a kind gift from Prof. F. Shao, National Institute of Biological Sciences, Beijing, China) associated with the protective antigen of *B. anthracis* (PA; 50 ng/ml, Calbiochem, San Diego, CA, USA); and for Pyrin inflammasome: TcdB (125 or 12.5 ng/ml, Abcam, Cambridge, UK) [9]. Following the incubation, cells were centrifuged and supernatants were collected.

### Cytokine detection and cell death assay

Levels of IL-1 $\beta$  in monocyte supernatants were quantified by ELISA (R&D Systems, Minneapolis, MN, USA), IL-1 $\beta$  values obtained for the most relevant stimuli are presented for each patient and the corresponding healthy controls in supplementary Table S1, available at *Rheumatology* Online. IL-18 ELISA was performed using anti-human IL-18 antibody (4 µg/ml; cat. no. D044-3, MBL,

Woburn, MA, USA) and anti-human IL-18 antibody coupled to biotin (20 ng/ml, cat. no. D045-6, MBL), respectively, as capture and detection antibodies.

Cell death was monitored by incubating  $2 \times 10^4$  monocytes per well of a black 96-well plate (Costar, Corning, Fisher Scientific, Waltham, MA, USA) with PI (Sigma-Aldrich) at 5 µg/ml. In preliminary experiments, we determined that this cell number was the lowest allowing a robust and reproducible cell death signal using PI incorporation. There were three technical replicates per condition. TcdB was added at 125 ng/ml in the absence of any priming signal. Nigericin was added at 5 µM after a 3 h priming with LPS at 10 ng/ml. Real time PI incorporation was measured every 5 min from 15 min to 105 or 135 min post-TcdB intoxication on a fluorimeter (Tecan, Männedorf, Switzerland) using the following wavelengths: excitation, 535 nm (bandwidth 15 nm); emission, 635 nm (bandwidth 15 nm) [29, 30].

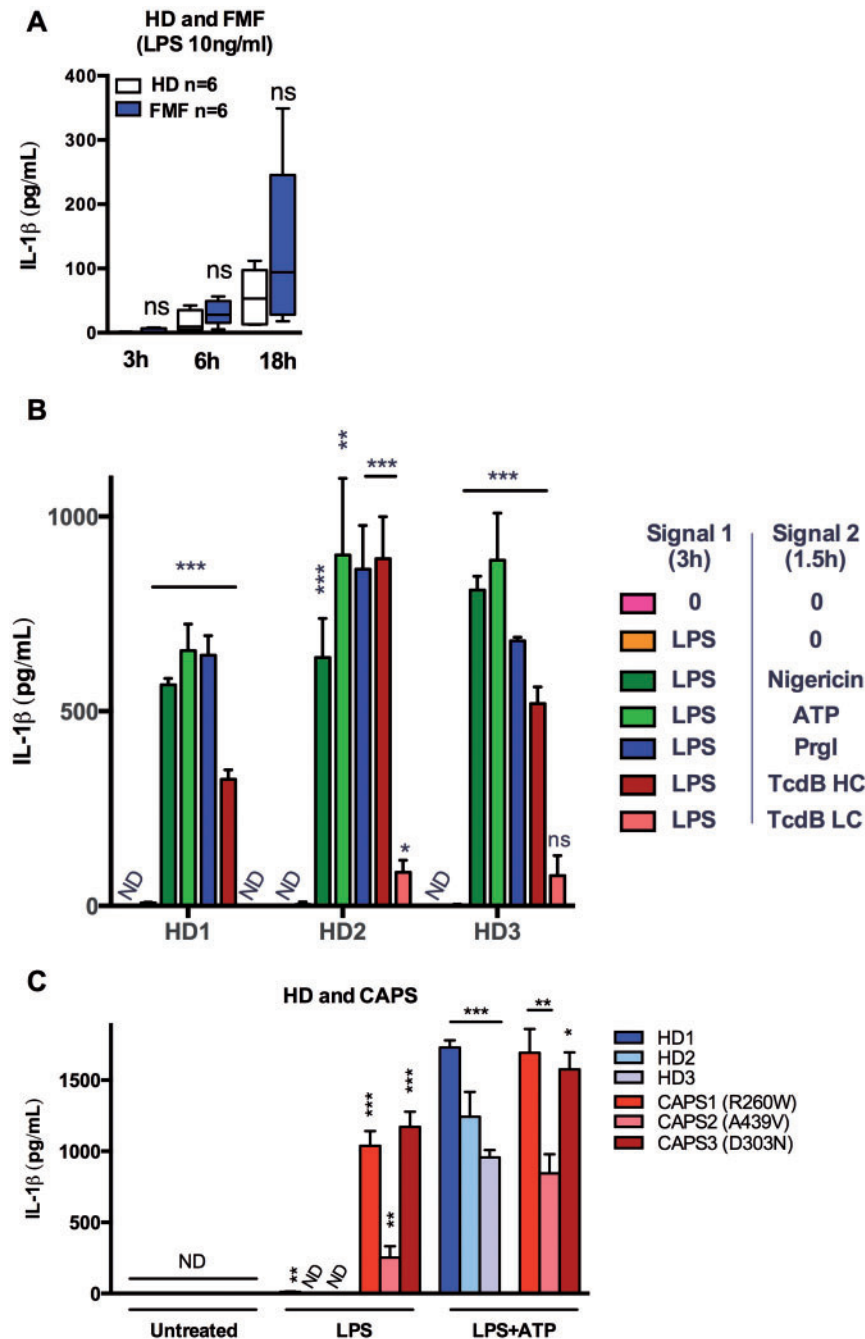
### Statistical analysis

Statistical analysis is described in the supplementary data, section Statistical analysis, available at *Rheumatology* Online.

## Results

### Development of a phenotyping platform to monitor the inflammasome activation in human monocytes

The canonical NLRP3 inflammasome activation requires two signals: a priming signal to induce proIL-1 $\beta$  and license the NLRP3 inflammasome [31, 32] and a specific signal to activate NLRP3. The addition of signal 2 is facultative in LPS-primed human monocytes [13, 14, 20, 33, 34] due to the gradual formation of non-canonical/alternative inflammasomes, which may involve caspase-4/5 and/or caspase-8 [13, 14]. As previously described [20], a long-term LPS treatment, which activates non-canonical/alternative inflammasomes [13], leads to higher IL-1 $\beta$  secretion by monocytes from FMF patients than by monocytes from HDs (Fig. 1A). In order to specifically assess the functionality of the various inflammasomes to respond to specific signals 2 in primary monocytes, we selected a short priming step (3 h) (Fig. 1B). Under such experimental conditions, and over a large range of cell number (supplementary Fig. S1, available at *Rheumatology* Online), a substantial IL-1 $\beta$  secretion was observed only upon addition of the specific NLRP3 (nigericin or ATP), NLRC4 (PA + LFn-PrgI) or Pyrin (TcdB) stimulus (Fig. 1B, supplementary Fig. S2, available at *Rheumatology* Online). To further exclude the possible confounding factors associated with the non-canonical/alternative inflammasomes, we also used another priming signal: MDP, a Nod2 ligand. MDP treatment does not activate the non-canonical/alternative inflammasomes [13, 14]. To monitor monocyte responses to a matrix of inflammasome stimuli and taking into account the limited volume of blood sample available from paediatric patients, we optimized our assay to use  $5 \times 10^3$  monocytes per condition (supplementary Fig. S1, available at *Rheumatology* Online). This cell number is

**Fig. 1** An optimized assay to study the canonical inflammasomes in monocytes

(A) Monocytes ( $5 \times 10^3$  per well) from HDs ( $n=6$ ) and FMF patients ( $n=6$ ) were primed for the indicated time with LPS (10 ng/ml). (B) Monocytes from HDs ( $n=3$ ) were primed for 3 h with LPS. NLRP3, NLRC4 and Pyrin inflammasomes were activated by incubation with their specific activating stimuli for 1.5 h: nigericin or ATP; PA + LFn-PrgI; TcdB at 125 ng/ml (HC) or 12.5 ng/ml (LC). (C) Monocytes from HD ( $n=3$ ) and patients with cryopyrin-associated periodic syndrome (CAPS,  $n=3$ ) were primed for 3 h LPS and when indicated stimulated with ATP for 1.5 h. *NLRP3* mutations are indicated.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns: not significant. ATP, adenosine triphosphate; CAPS, cryopyrin-associated periodic syndrome; HD: healthy donor; LFn: lethal factor N-terminal domain; LPS, lipopolysaccharide; ND: not detected; PA: protective antigen of *B. anthracis*; TcdB, *Clostridium difficile* toxin B.

typically at least 10-fold lower than what is typically used in the literature [13, 14]. While it may hinder the accurate detection of IL-1 $\beta$  secreted at early time points in the absence of signal 2 (Fig. 1A), the IL-1 $\beta$  levels obtained after addition of signals 1 and 2 were in the optimal range (500–1000 pg/ml) for ELISA detection (Fig. 1B). Furthermore, decreasing the monocyte number by a factor of 10 allows a symmetric 10-fold increase in the number of possible stimulations to be tested with the same blood volume.

We first assessed the ability of our assay to identify deviant inflammasome responses in primary monocytes by analysing the well-characterized response of monocytes from CAPS patients [35]. *NLRP3* gain-of-function mutations are known to trigger inflammasome activation in response to short priming with LPS in the absence of signal 2 [35]. Accordingly, in our assay, monocytes from three CAPS patients harbouring different mutations released high levels of IL-1 $\beta$  upon LPS priming only. In contrast, in our experimental conditions, addition of a signal 2 was required to trigger detectable IL-1 $\beta$  release from HD monocytes (Fig. 1C).

We then evaluated the response of monocytes from FMF patients treated with signal 1 only. In our experimental conditions, monocytes from FMF patients and HD released low to undetectable levels of IL-1 $\beta$  when stimulated only with the priming signal for 3 h (LPS or MDP alone—Fig. 1A, supplementary Figs S1B and S3, available at *Rheumatology* Online). As previously observed using PBMCs [21, 36], this result indicates that the Pyrin inflammasome is not constitutively activated in FMF patients. While CAPS and FMF are both classified as inflammasomopathies [37], the molecular mechanisms underlying the disease are thus clearly different.

#### Monocytes from FMF patients display a specific increased response to low doses of a Pyrin-activating stimulus relative to monocytes from HD

We analysed the monocyte response of three FMF patients and three HDs using a short priming step and various doses of ATP, PA + LFn-PrG1 and TcdB to engage the NLRP3, NLRC4 and Pyrin inflammasomes, respectively (Fig. 2A). At the high TcdB concentrations (>250 ng/ml), monocytes from HDs and FMF patients secreted similar levels of IL-1 $\beta$ . This result demonstrates that FMF-associated *MEFV* mutations do not impair the ability of Pyrin to detect bacterial toxin-mediated RhoGTPases modifications. This experiment confirms a very recent report [21] and further rules out the hypothesis that FMF mutations may be loss-of function mutations [19, 20, 38].

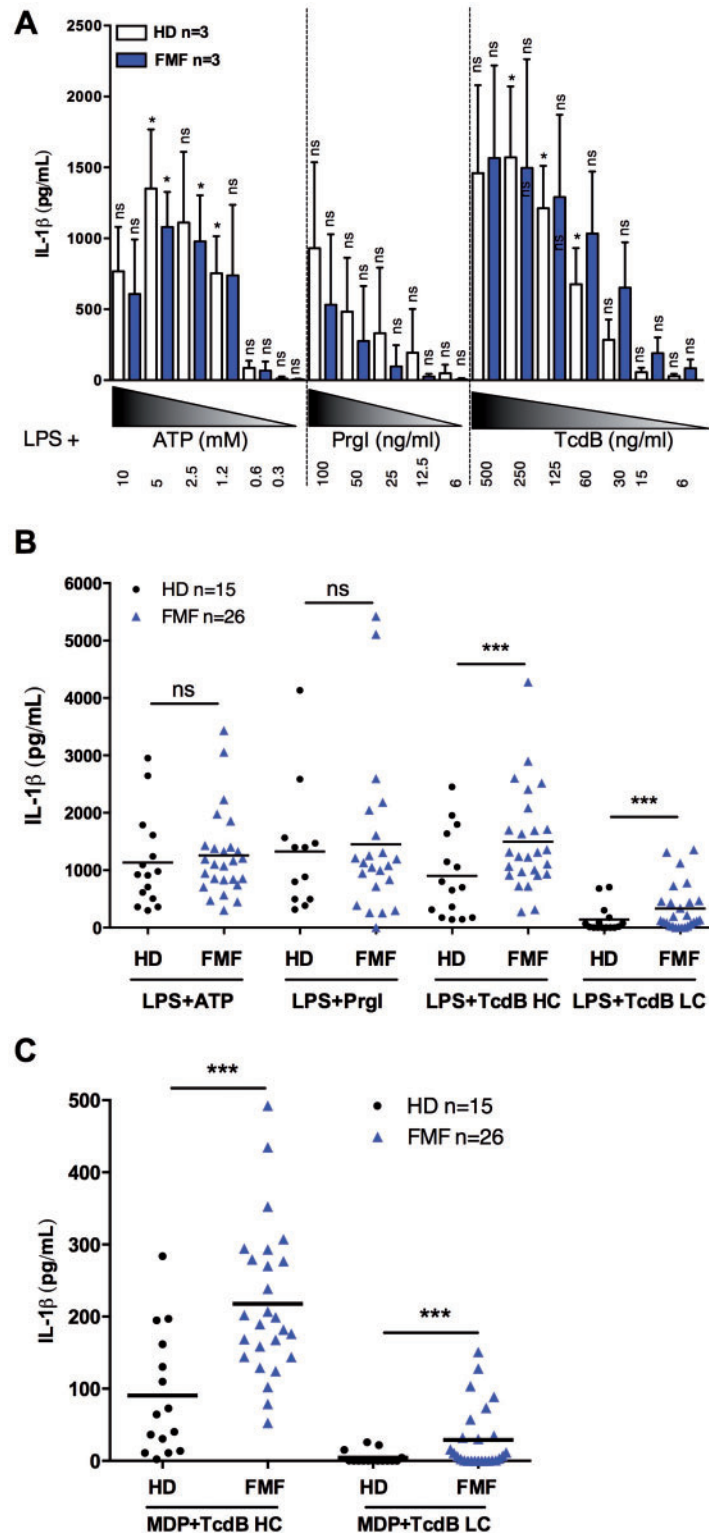
Interestingly, we observed that monocytes from FMF patients sustained relatively strong IL-1 $\beta$  production at low TcdB doses, while in monocytes from HDs, IL-1 $\beta$  secretion decreased sharply with decreasing TcdB concentrations (Fig. 2A). In contrast, upon engagement of NLRP3 or NLRC4 inflammasomes, the dose responses of primary monocytes from HDs and FMF patients were similar (Fig. 2A). These results suggest that FMF-associated *MEFV* mutations, far from impairing the ability of Pyrin to

detect bacterial toxin-mediated RhoGTPase modifications, may decrease specifically the activation threshold of the Pyrin inflammasome. To strengthen this finding, we selected two TcdB concentrations in the range in which we observed a deviating response between HD and FMF patients (HC: 125 ng/ml and LC: 12.5 ng/ml) and analysed IL-1 $\beta$  secretion in a large cohort of FMF patients and HDs. Confirming the previous analysis, monocytes from FMF patients secreted significantly higher IL-1 $\beta$  levels than HD monocytes in response to LPS + TcdB (Fig. 2B). This result held true when monocytes were primed with MDP (Fig. 2C) indicating that the observed differences were unrelated to the nature of the priming signal but, rather, specific to the Pyrin inflammasome-activating stimulus. We did not observe any correlation between IL-1 $\beta$  levels following TcdB treatment and SAA/CRP levels, clinical manifestations or colchicine treatment. However, most of the included patients were under colchicine treatment and clinically asymptomatic at the time of blood collection, possibly masking a correlation between the clinical and the monocyte Pyrin inflammasome phenotypes.

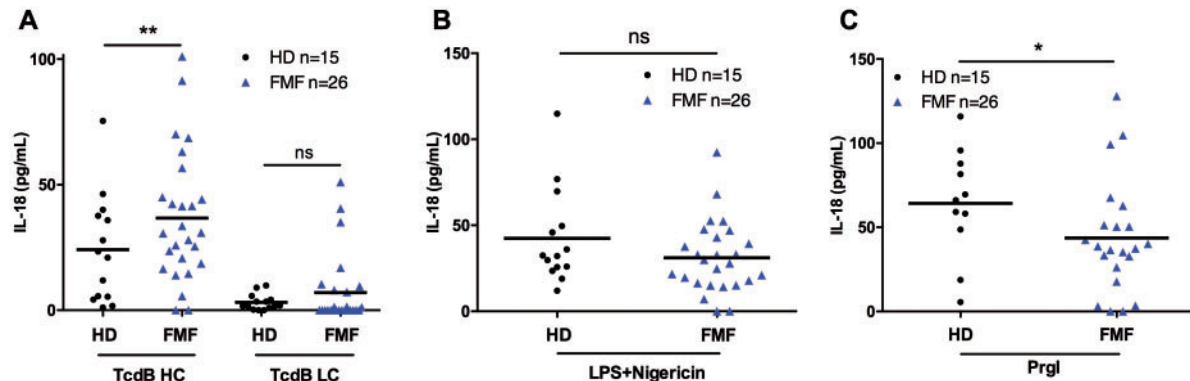
Using a short LPS or MDP priming step and upon stimulation of the NLRP3 or NLRC4 inflammasomes, we did not observe any statistical differences in IL-1 $\beta$  secretion between HD and FMF patients (Fig. 2A and B; supplementary Fig. S3B, available at *Rheumatology* Online) demonstrating that the increased inflammasome response observed in monocytes from FMF patients is Pyrin specific.

To further investigate these Pyrin-specific responses, we studied another inflammasome-dependent cytokine: IL-18. In monocytes, proIL-18 is constitutively expressed. Pyrin and NLRC4 inflammasomes do not require a priming step. We thus monitored IL-18 release following direct addition of Pyrin- or NLRC4-activating stimuli. Monocytes from FMF patients secreted higher levels of IL-18 compared with monocytes from HDs in response to TcdB (Fig. 3A). In contrast, engagement of NLRP3 or NLRC4 inflammasomes led to a minor decrease in IL-18 secretion by monocytes from FMF patients compared with HDs (Fig. 3B and C) further highlighting the Pyrin specificity of the increased inflammasome response.

Finally, while inflammasome-mediated cytokines are likely central to FMF symptoms (as demonstrated by the use of IL-1 blockers [39, 40] and in mouse models [19, 38]), caspase-1 activation also triggers an inflammatory cell death termed pyroptosis. Pyroptosis is associated with a very fast membrane integrity rupture, which can be monitored by following PI entry into cells and the corresponding increase in PI fluorescence [29, 30]. In seven independent experiments, monocytes from 12 out of 13 FMF patients displayed faster cell death kinetics in response to TcdB than monocytes from HD (see Fig. 4A and B for two representative experiments and Fig. 4C for a kinetics including all the patients). To obtain quantitative data from each independent kinetics, we calculated the area under the curve (Fig. 4E) and the time post-TcdB addition required to reach 20% cell death (Fig. 4G). On average, monocytes from FMF patients were 20% dead by 90 min post-intoxication. In contrast,

**Fig. 2** Pyrin inflammasome activity is specifically heightened in monocytes from FMF patients relative to healthy donors

(A) Monocytes from HDs (open bars,  $n=3$ ) and FMF patients (blue bars,  $n=3$ ) were treated for 3 h with LPS and decreasing doses of ATP, PA + LFn-Prgl and TcdB. (B and C) Monocytes from HDs ( $n=15$ ) and FMF patients ( $n=26$ ) were primed for 3 h with LPS or muramyl dipeptide (MDP) and incubated for 1.5 h with ATP, PA + LFn-Prgl and TcdB at 125 ng/ml (HC) or 12.5 ng/ml (LC). Each symbol represents the average value of three technical replicates from a single HD (back circle) or FMF patient (blue triangle). The bar represents the mean (A–C). IL-1 $\beta$  production was assessed by ELISA in the supernatants. \* $P < 0.05$ ; \*\*\* $P < 0.001$ . ATP, adenosine triphosphate; HD: healthy donor; LPS, lipopolysaccharide; LFn: lethal factor N-terminal domain; PA: protective antigen of *B. anthracis*; TcdB, *Clostridium difficile* toxin B.

**Fig. 3** IL-18 production is increased specifically upon Pyrin inflammasome activation, independently of the priming signal

Monocytes from HDs (n = 15) and FMF patients (n = 26) were left unprimed (**A** and **C**) or primed for 3 h with 10 ng/ml LPS (**B**) and treated for 1.5 h with TcdB at 125 ng/ml (HC) or 12.5 ng/ml (LC) (**A**); 5  $\mu$ M nigericin (**B**); 50 ng/ml PA + LFn-Prgl. Supernatants were collected. IL-18 production was assessed by ELISA. Each symbol represents the average value of three technical replicates from a single HD (black circle) or FMF patient (blue triangle). The bar represents the mean. Wilcoxon's matched-pairs test was performed, and two-tailed P-values comparing HD and FMF are shown. \*P < 0.05; \*\*P < 0.01; ns: not significant. HD: healthy donor; LPS, lipopolysaccharide; LFn: lethal factor N-terminal domain; PA: protective antigen of *B. anthracis*; TcdB, *Clostridium difficile* toxin B.

monocytes from most of the healthy controls did not reach 20% cell death at the end of our kinetics (135 min post-intoxication) (Fig. 4G). Once again the faster cell death was specific for the Pyrin inflammasome stimulus TcdB. Indeed, monocytes from FMF patients died with the same kinetics as monocytes from HDs upon stimulation with LPS + nigericin (Fig. 4D, F and H). These results show that monocytes from FMF patients not only produce more cytokines in response to low doses of TcdB but also die faster, demonstrating a higher reactivity of the Pyrin inflammasome in FMF patients.

Altogether these functional analyses demonstrate that, under short priming conditions, FMF-associated *MEFV* mutations decrease the activation threshold of the Pyrin inflammasome without altering NLRP3 or NLRC4 inflammasome responses.

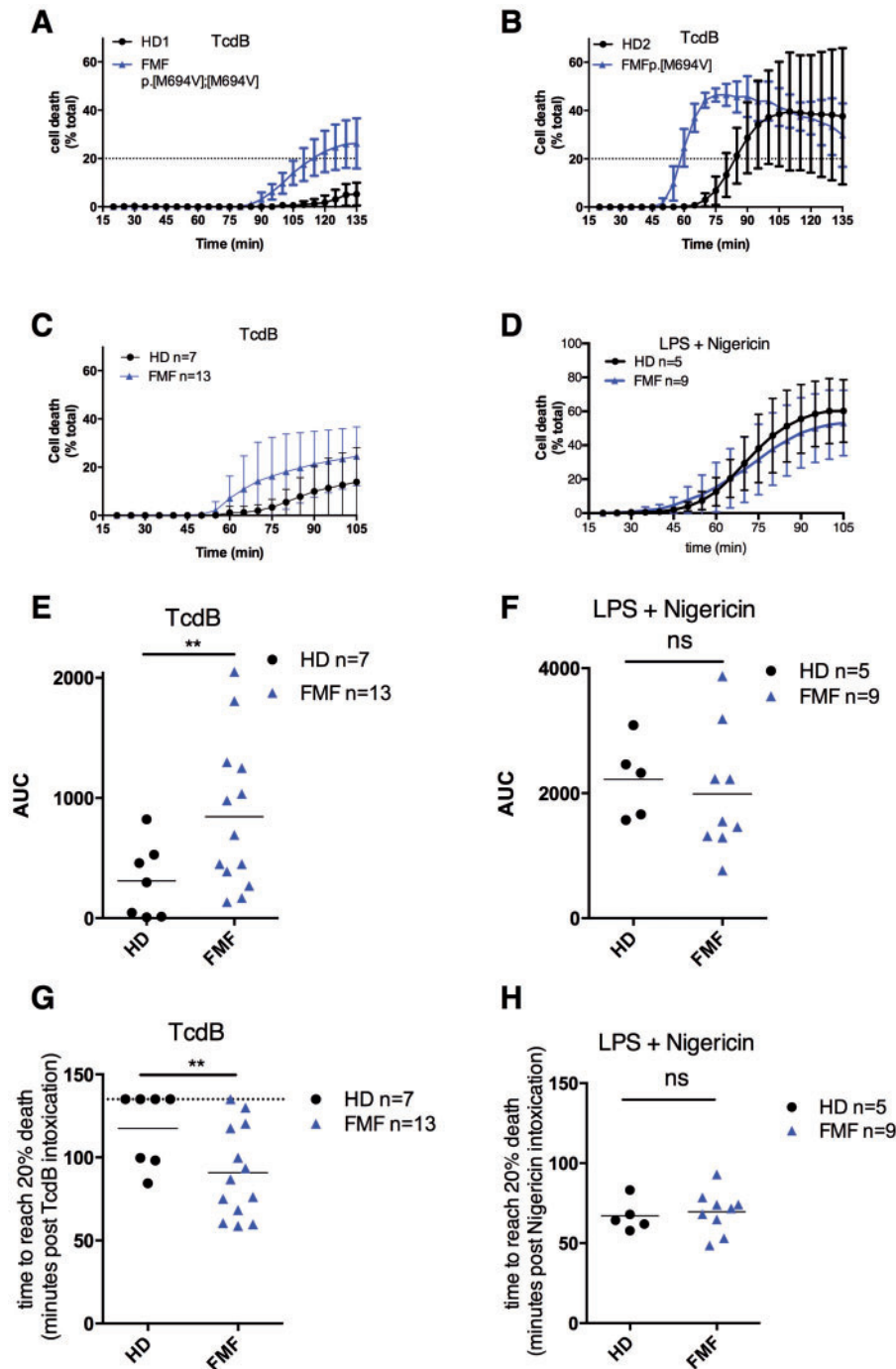
The pyrin inflammasome response is exacerbated in monocytes from p.M694V homozygous patients compared with monocytes from p.M694V heterozygous patients

The severity of FMF disease is linked to the nature of the *MEFV* mutation and to the number of mutated alleles [22]. To test whether this correlates with the *in vitro* strength of the Pyrin inflammasome responses, we compared the response of monocytes from FMF patients carrying one or two copies of the most prevalent and highly penetrant mutation in exon 10 (p.M694V variant). Of note, the other variants were not frequent enough in our cohort to draw any significant conclusions. Monocytes from patients carrying the p.M694V allele at the homozygous state released significantly more IL-1 $\beta$  (Fig. 5A) than heterozygous monocytes upon stimulation with TcdB. Importantly, this increased response was specific to the Pyrin inflammasome since no statistical differences were

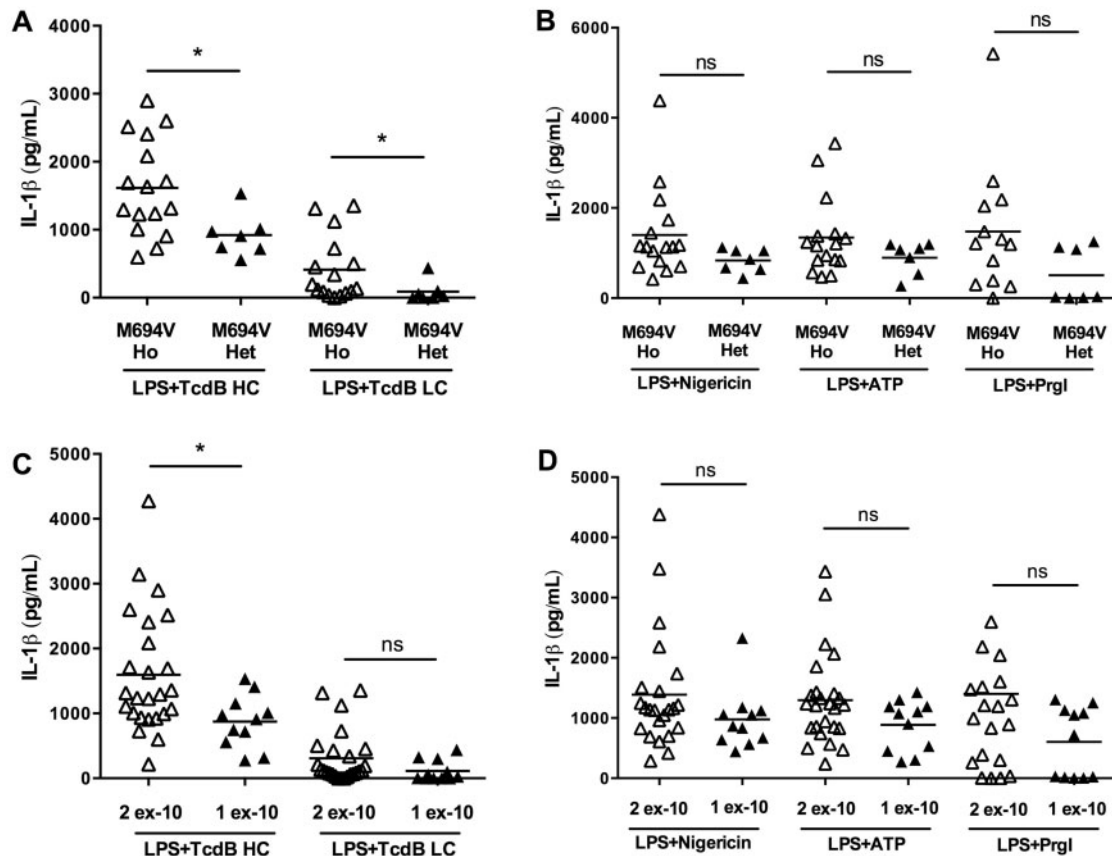
observed following stimulation of the NLRP3 and NLRC4 inflammasomes (Fig. 5B). Upon engagement of the NLRP3 and NLRC4 inflammasome, IL-1 $\beta$  levels released by monocytes from heterozygous patients tended to be lower than IL-1 $\beta$  levels released by monocytes from homozygous patients. Although non-significant, this difference, which is most striking upon NLRC4 engagement (average IL-1 $\beta$  levels: 1478, 505 and 1327 pg/ml, respectively, for homozygous, heterozygous FMF patients and HDs), was mainly due to decreased IL-1 $\beta$  responses of heterozygous monocytes compared with monocytes from HDs. Pyrin-specific differences were also observed when considering patients carrying a single or two exon-10 pathogenic variants [6, 7] (Fig. 5C and D). This result demonstrates that the Pyrin inflammasome responses in monocytes are modulated by the copy number of pathogenic *MEFV* mutations.

## Discussion

The description of Pyrin as an inflammasome component sensing RhoGTPase modifications [9] gave us the opportunity to investigate the functional consequences of *MEFV* mutations in FMF patients. Using a short priming step and a matrix of inflammasome stimuli, we demonstrated that FMF-associated Pyrin variants trigger increased inflammasome responses to low concentrations of a Pyrin-activating stimulus but not to NLRP3- or NLRC4-activating stimuli. Our results indicate that FMF mutations are gain-of-function mutations [19]. In contrast to the gain-of-function mutations of *NLRP3* in CAPS patients (Fig. 1B), of *NLRC4* in NLRC4-macrophage activation syndromes [17] and of *MEFV* (p.S242R) in Pyrin-associated autoinflammation with neutrophilic dermatosis [18], we did not observe IL-1 $\beta$  secretion in response to a short treatment with LPS only. In agreement with previous data

**Fig. 4** Monocytes from FMF patients die faster than healthy donors monocytes upon TcdB treatment

Monocytes from FMF patients and HDs were treated with TcdB or LPS + nigericin in the presence of propidium iodide (PI). (A and B) Two representative experiments comparing one FMF patient and one HD. (C and D) Means (s.d.) from HDs and FMF patients are shown. (E and F) Each symbol represents the AUC (15–105 min) from a single HD or FMF patient. (G and H) Each symbol represents the time post-TcdB or post-nigericin intoxication to reach 20% cell death from a single HD or FMF patient. The horizontal dotted line corresponds to 135 min. \*\* $P < 0.01$ ; ns: not significant. AUC: area under the curve; HD: healthy donor; LPS, lipopolysaccharide; TcdB, *Clostridium difficile* toxin B.

**Fig. 5** Pyrin inflammasome responses from patients bearing one or two *MEFV* mutations reveal a gene-dosage effect

Monocytes from FMF patients homozygous (**A** and **B**:  $n = 14$ ; open triangles) or heterozygous (**A** and **B**:  $n = 7$ ; black triangles) for the M694V mutation of *MEFV* (**A** and **B**) or carrying two (**C** and **D**:  $n = 24$ ; open triangles) or a single (**C** and **D**:  $n = 11$ ; black triangles) *MEFV* allele(s) containing an exon 10 pathogenic mutation [6] (**C** and **D**) were primed with LPS and inflammasomes were activated by addition of TcdB (HC = 125 ng/ml; LC = 12.5 ng/ml), nigericin, ATP or PA + LFn-Prgl. Supernatants were collected, and IL-1 $\beta$  production was assessed by ELISA. The bar represents the mean. Unpaired Mann-Whitney tests were performed, and two-tailed P-values comparing FMF patients are shown (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ; ns: not significant). 1 ex-10, patients carrying one mutated allele in the exon 10 of *MEFV*; 2 ex-10, patients carrying two mutated alleles in the exon 10 of *MEFV*. ATP, adenosine triphosphate; HD: healthy donor; Het: heterozygous; Ho: homozygous; LPS, lipopolysaccharide; LFn: lethal factor N-terminal domain; PA: protective antigen of *B. anthracis*; TcdB, *Clostridium difficile* toxin B.

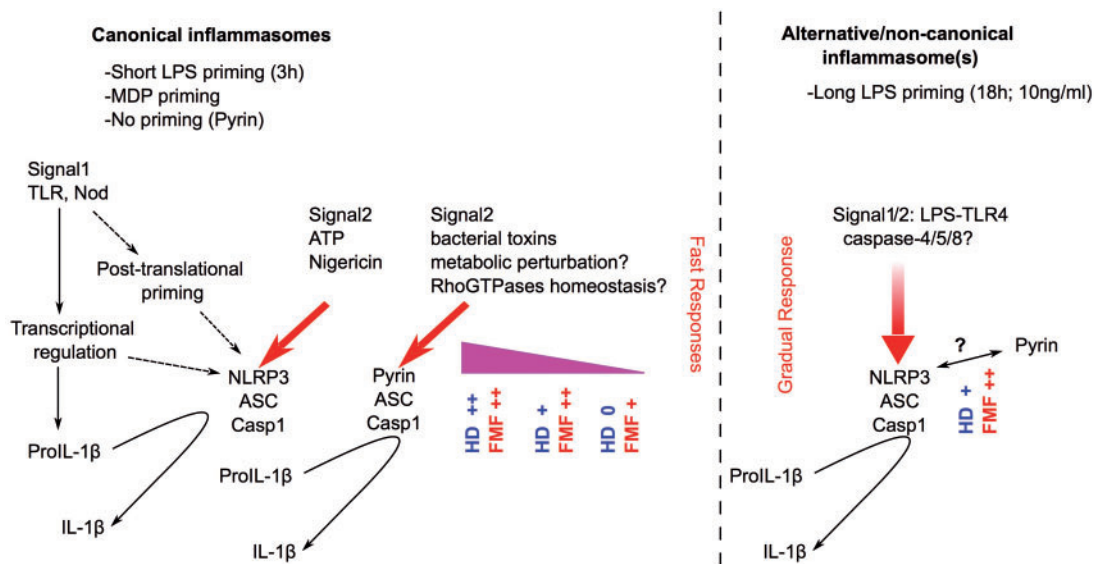
obtained on PBMCs [21, 22], our data thus confirm that FMF mutations are not classical gain-of-function mutations leading to a constitutively active inflammasome. Rather, we demonstrated that FMF mutations are hyper-morphic mutations that specifically decrease the activation threshold of the Pyrin inflammasome. It is currently unclear whether the increased activity of the Pyrin variants results from the loss of a negative regulation or from a facilitated activation mechanism.

A concurrent study recently identified that FMF mutations lift the obligatory requirement for microtubules in Pyrin inflammasome activation [21]. While they did not observe any differences in IL-1 $\beta$  secretion upon *C. difficile* toxin treatment between HDs and FMF patients, the dose of TcdB used in their study (1  $\mu$ g/ml) was higher than the range of concentrations (125–7 ng/ml) in which we observed

a difference in the response of FMF patients vs HDs. Similarly to their results, we observed that at high concentrations of toxins (TcdB > 250 ng/ml), IL-1 $\beta$  responses were similar between HDs and FMF patients. The poorly understood microtubule-dependent step required to activate the Pyrin inflammasome in HDs may be particularly important to set the threshold of activation of the Pyrin inflammasome in HDs. The absence of this regulatory mechanism in FMF patients may thus facilitate the response of FMF-associated Pyrin variants to low doses of TcdB. In contrast, high doses of TcdB might overrule the selective microtubule-related advantage of FMF-associated Pyrin variants leading to similar activation in FMF patients and HDs. Further studies are needed to explore this hypothesis.

Increased NLRP3-dependent IL-1 $\beta$  responses to long-term LPS exposure in monocytes from FMF patients

**Fig. 6** Model integrating the canonical and alternative/non-canonical inflammasomes and their dysfunction in FMF patients



NLRP3 and Pyrin canonical inflammasomes are typically monitored following a short-term priming. Detection of signal 2 leads to a fast inflammasome activation. TcdB triggers a similar response in HDs and FMF patients at high concentrations but at lower concentration triggers an increased response in FMF patients. Similarly, endogenous signals, providing weak Pyrin stimulation, may lead to increased inflammasome activation in FMF patients. For non-canonical/alternative inflammasomes, long-term LPS treatment provides both signals 1 and 2, resulting in a gradual NLRP3 activation. In such conditions, monocytes from FMF patients secrete more IL-1 $\beta$  than monocytes from HDs (adapted from [13]). Of note, lower IL-1 $\beta$  levels have been observed for shorter LPS treatments. However, IL-1 responses by monocytes of HDs and FMF patients were not statistically different at early time points. ASC, apoptosis-associated speck-like protein containing a CARD; ATP, adenosine triphosphate; Casp1: caspase-1; HD: healthy donor; LPS, lipopolysaccharide; MDP: muramyl dipeptide; Nod, nucleotide-binding oligomerization domain containing 1/2; TcdB, *Clostridium difficile* toxin B; TLR: Toll-like receptor.

compared with HDs have been previously described [20], a finding which we reproduced here (Fig. 1A). These conditions are likely to trigger the recently described alternative/non-canonical inflammasome complexes [13, 14]. LPS treatment modifies Rho GTPase activity [41] thus providing a possible molecular explanation for the role of Pyrin in monocytes exposed to LPS only. The role of Pyrin in the context of long-term LPS treatment and of alternative/non-canonical inflammasomes remains to be investigated (see model in Fig. 6). Using a short priming step to avoid the detection of this alternative inflammasome, we identified FMF-specific, Pyrin-dependent, NLRP3-independent phenotypes. Our data dovetail with findings obtained in mouse models of FMF indicating primarily NLRP3-independent phenotypes [19].

Importantly, we observed that the magnitude of the Pyrin inflammasome response is higher in patients bearing two *MEFV* exon 10 mutations compared with patients with a single *MEFV* exon 10 mutation. This result indicates a gene-dosage effect, which correlates with the previously described impact of *MEFV* gene dosage on the severity of FMF clinical manifestations [22].

Our work identifies FMF-associated *MEFV* mutations as hypermorphic mutations that allow the Pyrin inflammasome

to react faster (Fig. 4) and stronger (Fig. 2) than the Pyrin inflammasome from HD to low doses of RhoGTPase-modifying toxins. The high prevalence of *MEFV* mutations in the Mediterranean basin has long been suggested to be the result of a selective advantage for the human host to combat an as-yet-unknown pathogen [42]. Accordingly, numerous pathogens, including *C. difficile* and *Yersinia pestis* are sensed via the Pyrin inflammasome [10, 11]. Our present work demonstrating a reduced threshold of activation of the Pyrin inflammasome in FMF patients strongly supports the hypothesis of a selective advantage associated with the FMF Pyrin variants to fight these pathogens.

Finally, while we used a bacterial toxin as an experimental tool to specifically activate the Pyrin inflammasome, the nature of the Pyrin-engaging stimulus that leads to the cardinal symptoms in FMF patients is still uncharacterized. Various endogenous signals affect RhoGTPase homeostasis and, thus, are possible activators of the Pyrin inflammasome [43–46]. These endogenous signals are likely weak activators of the Pyrin inflammasome and low concentrations of toxins such as the ones used in our study may thus best recapitulate the physiological Pyrin stimulation conditions observed in natural settings. One can speculate that, in FMF patients, such endogenous

signals activate the Pyrin inflammasome due to its reduced threshold of activation (Fig. 6).

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## Supplementary data

Supplementary data are available at *Rheumatology* Online.

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