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

Hanne Van Gorp<sup>a,b,1</sup>, Pedro H. V. Saavedra<sup>a,b,1</sup>, Nathalia M. de Vasconcelos<sup>a,b</sup>, Nina Van Opdenbosch<sup>a,b</sup>, Lieselotte Vande Walle<sup>b</sup>, Magdalena Matusiak<sup>a,b</sup>, Giusi Prencipe<sup>c</sup>, Antonella Insalaco<sup>c</sup>, Filip Van Hauwermeiren<sup>a,b</sup>, Dieter Demon<sup>a,b</sup>, Delfien J. Bogaert<sup>d,e,f</sup>, Melissa Dullaers<sup>d,g</sup>, Elfride De Baere<sup>b</sup>, Tino Hochepiel<sup>j,k</sup>, Joke Dehoorne<sup>l</sup>, Karim Y. Vermaelen<sup>b,k</sup>, Filomeen Haerynck<sup>d,e,f</sup>, Fabrizio De Benedetti<sup>c</sup>, and Mohamed Lamkanfi<sup>a,b,2</sup>

<sup>a</sup>Inflammation Research Center, VIB, Zwijnaarde, B-9052, Belgium; <sup>b</sup>Department of Internal Medicine, Ghent University, Ghent, B-9000, Belgium;<br><sup>c</sup>Rheumatology Unit, Bambino Gesù Children’s Hospital, Rome, I-00146, Italy; <sup>d</sup>Clinical Immunology Research Laboratory, Centre for Primary Immunodeficiency Ghent, Ghent University Hospital, Ghent, B-9000, Belgium; <sup>e</sup>Department of Pediatric Immunology and Pulmonology, Centre for Primary Immunodeficiency Ghent, Ghent University Hospital, Ghent, B-9000, Belgium; <sup>f</sup>Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, B-9000, Belgium; <sup>g</sup>Laboratory of Immunoregulation, Inflammation Research Center, VIB, Zwijnaarde, B-9052, Belgium; <sup>h</sup>Center for Medical Genetics Ghent, Ghent University, Ghent, B-9000, Belgium; <sup>i</sup>Department of Biomedical Molecular Biology, Ghent University, Ghent, B-9000, Belgium; <sup>j</sup>Department of Pediatric Rheumatology, Ghent University Hospital, Ghent, B-9000, Belgium; and <sup>k</sup>Tumor Immunology Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Ghent, B-9000, Belgium

Edited by Vishva M. Dixit, Genentech, San Francisco, CA, and approved October 28, 2016 (received for review August 8, 2016)

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 inflammasome activation and secretion of proIL-1β and proIL-18 has been reported to date (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 InFevs 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 here reports that Clostridium difficile and its enterotoxin A (TcdA) engage the Pyrin inflammasome. Moreover, activation of the Pyrin inflammasome, but not other inflammasomes, was hampered by microtubule-depolymerizing drugs in mice 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.

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.


Conflict of interest statement: H.V.G., P.H.V.S., and M.L. are listed as inventor on a patent application on immunological FMF diagnosis.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

1H.V.G. and P.H.V.S. contributed equally to this work. 2To whom correspondence should be addressed. Email: mohamed.lamkanfi@irc.vib-ugent.be.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1613156113/-/DCSupplemental.
here expands the set of Pyrin inflammasome-activating agents to live *C. difficile* infection and its enterotoxin TcdA. We further show that among the different known inflammasome sensors, wild-type Pyrin of both humans and mice relies selectively on microtubules for inflammasome activation. Microtubules control Pyrin signaling downstream of Pyrin dephosphorylation. Surprisingly, however, we found that FMF mutations lift the obligatory requirement for microtubules in activating the Pyrin inflammasome, providing a conceptual framework for understanding FMF and enabling immunological segregation of FFM from related autoinflammatory disorders.

**Results**

**TcdA Activates the Pyrin Inflammasome in Mouse Macrophages and Human Monocytes.** The Pyrin inflammasome responds to infection with *Burkholderia cenocepacia* (12, 13) and the Rho GTPases-selectively Inhibit the Pyrin inflammasome. Regardless, *C. difficile*-infected PBMCs of healthy individuals also secreted high levels of IL-1β and IL-18, and these responses were inhibited by the cell-permeable caspase-1 inhibitor Ac-YVAD-cmk (Fig. S3 G and H). As in murine macrophages, *C. difficile*-induced inflammasome activation in human PBMCs was elicited by the bacterial toxins because IL-1β and IL-18 secretion was blunted upon infection with the TcdA/TcdB-deficient *C. difficile* mutant (Fig. S3 J). Together, we show that TcdA and TcdB are fully responsible for *C. difficile*-induced inflammasome activation in rodents and humans alike.

**LPS Priming Is Dispensable but Enhances Pyrin Inflammasome Activation.** Having established that the enterotoxins are required for inflammasome activation induced by *C. difficile* infection, we next set out to investigate the mechanisms by which the enterotoxins engage the Pyrin inflammasome. We first asked whether Pyrin activation required priming, an important hallmark of the Nlrp3 inflammasome (23). Both TcdA and TcdB activated caspase-1 maturation in LPS-primed BMDMs but not caspase-11 (Fig. S1 C and D). Consistent with TcdA activating a canonical inflammasome, extracellular release of IL-1β continued unabated in BMDMs lacking caspase-11 but was blunted in ASC−/− macrophages, in caspase-1−/− caspase-11−/− BMDMs, and in macrophages of a newly generated caspase-1−/−deficient mouse strain (Fig. S1D and Fig. S2). TcdA paralleled TdB in engaging the Pyrin inflammasome because TcdA-induced caspase-1 activation and subsequent cleavage of proIL-1β were abolished in Pyrin (Mefv−/−) BMDMs but not in Nlrp3−/− macrophages but not in Nlrp3−/− macrophages (Fig. S1E). Consistent with these findings, TcdA-intoxicated macrophages required Pyrin, but not Nlrp3, for secreting IL-1β (Fig. S1F). These results demonstrate that TcdA selectively activates the Pyrin inflammasome in murine BMDMs. To examine whether this selective activation also occurs in humans, PBMCs from three healthy donors were incubated with the cell-permeable caspase-1 inhibitor Ac-YVAD-cmk or the Nlrp3 inflammasome-selective inhibitor MCC950/CRID3 (17, 18) before intoxication with TcdA. Pharmacological inhibition of caspase-1 significantly reduced TcdA-induced cleavage of proIL-1β and the extracellular release of IL-1β and IL-18 from human PBMCs (Fig. S1 G–I). In contrast, TcdA-induced inflammasome activation was insensitive to MCC950/CRID3 at drug concentrations that effectively blunted Nlrp3−/−-reared IL-1β and IL-18 secretion from nigericin-treated PBMCs (Fig. S1 G–K). Thus, our results confirm recent findings that purified TcdA selectively engages the Pyrin inflammasome in mouse BMDMs (19) and extend these results to human PBMCs.

**The Pyrin Inflammasome Is Engaged by *C. difficile Infection.**** Both TcdA and TcdB contribute critically to *C. difficile*-induced pseudomembranous colitis (16), although the pathogen also activates additional immune mechanisms independently of TcdA and TcdB (20). Because microbial pathogens may express several virulence factors that engage multiple inflammasomes in parallel (21, 22), and because the mechanisms of inflammasome activation induced by *C. difficile* infection are unknown, we next studied inflammasome responses in *C. difficile*-infected macrophages. Caspase-1 was activated in wild-type BMDMs infected with *C. difficile*, and this activation resulted in substantial cleavage and extracellular release of mature IL-1β (Fig. S3 A and B). These responses required live bacteria and the expression of the bacterial toxins TcdA and TcdB because inflammasome-dependent cytokine processing and secretion were blunted when BMDMs were exposed to heat-killed *C. difficile* or were infected with the TcdA/B-deficient (VP11186) *C. difficile* strain (Fig. S3 A and B). As with the purified toxins (Fig. S1), *C. difficile* infection-induced caspase-1 activation required Pyrin and the inflammasome adaptor ASC, whereas Nlrp3 and caspase-11 were dispensable (Fig. S3 C and D). Likewise, Mefv−/− and Asc−/− BMDMs failed to secrete IL-1β in the culture supernatants, whereas the supernatants of *C. difficile*-infected Nlrp3−/− and caspase-11−/− macrophages contained significant levels of IL-1β (Fig. S3 E and F). Notably, caspase-1 was responsible for the gross amount of IL-1β maturation and secretion, but proIL-1β maturation and IL-1β secretion were not fully inhibited in caspase-1−/− caspase-11−/− macrophages (Fig. S3 C and E). These results suggest that additional proteases may, to a limited extent, contribute to Pyrin- and ASC-dependent IL-1β secretion in *C. difficile*-infected macrophages. Regardless, *C. difficile*-infected PBMCs of healthy individuals also secreted high levels of IL-1β and IL-18, and these responses were inhibited by the cell-permeable caspase-1 inhibitor Ac-YVAD-cmk (Fig. S3 G and H). As in murine macrophages, *C. difficile*-induced inflammasome activation in human PBMCs was elicited by the bacterial toxins because IL-1β and IL-18 secretion was blunted upon infection with the TcdA/TcdB-deficient *C. difficile* mutant (Fig. S3 J). Together, we show that TcdA and TcdB are fully responsible for *C. difficile*-induced inflammasome activation in rodents and humans alike.

**Microtubule-Depolymerizing Drugs Selectively Inhibit the Pyrin Inflammasome.** Ectopically expressed Pyrin partially associates with cytoskeletal structures (30), but how this localization relates to its physiologic role in inflammasome activation is unclear. We found that neither inhibition of actin polymerization with cytochalasin D nor small-molecule targeting of downstream actin effectors (c-Abl kinase with STI-571, myosin II with myostatin, and ROCK 1/2 with Y-27632) interfered substantially with Pyrin-TcdA interaction because IL-1β secretion from murine BMDMs and human PBMCs (Fig. S5 A and B). Also the microtubule-stabilizing agent paclitaxel (taxol) failed to modulate the secretion of IL-1β and IL-18 from TcdA-treated PBMCs (Fig. S5 C and D). In marked contrast, however, the microtubule polymerization inhibitor...
Differential microtubule regulation of Pyrin inflammasome activation

Van Gorp et al.

Microtubule depolymerizing drugs specifically inhibit the Pyrin inflammasome. (A and B) Wild-type LPS-primed BMDMs were pretreated with lumiocolchicine or colchicine before stimulation with TcdA. Samples were immunoblotted for caspase-1 and IL-1β (A), and supernatants were analyzed for IL-1β (B). (C) Unprimed BMDMs were pretreated with colchicine before infection with C. difficile followed by supernatant collection and analysis of IL-1β. (D–F) PBMCs from healthy donors (n = 3) were pretreated with lumiocolchicine or colchicine before stimulation with TcdA. Samples were immunoblotted for IL-1β (D), and supernatants were analyzed for IL-1β (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, nocardazole, ABT-751, CA4P, or CYT997 before stimulation with TcdA. Samples were immunoblotted for caspase-1 and IL-1β (G), and supernatants were analyzed for IL-1β (H). PBMC samples were immunoblotted for IL-1β (I), and supernatants were analyzed for IL-1β (J) and IL-18 (K). (L–O) LPS-primed BMDMs were pretreated with colchicine before being stimulated with activators of the Nlpr1b (anthrax lethal toxin; LeTx) (L), Nlrc4 (S. Typhimurium; STm) (M), AIM2 (dsDNA) (N), and Nlpr3 (nigericin; Nig) (O) inflammasomes followed by immunoblot to detect caspase-1 and IL-1β. Black arrowheads indicate procaspase-1 and proIL-1β, and white arrowheads indicate the p20 and p17 subunits. Luminex data are shown as mean ± 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-1β from TcdA-treated BMDMs (Fig. 1 A and B). Lumiocolchicine, a structurally related colchicine photosomer 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-1β secretion from C. difficile-infected BMDMs (Fig. 1C). Consistent with these results, colchicine, but not lumiocolchicine, inhibited Pyrin-induced proIL-1β maturation in human PBMCs (Fig. 1D) and thus prevented the secretion of IL-1β 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-1β from TcdA-treated BMDMs (Fig. 1 G and H). Parallelizing these results, these tubulin polymerization inhibitors prevented Pyrin-dependent IL-1β maturation in human PBMCs (Fig. 1I). Similarly, secretion of IL-1β 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 Nlpr1b-dependent auto-maturation of caspase-1 and cleavage of proIL-1β regardless of whether BMDMs had been pretreated with colchicine (Fig. 1L). Nlrc4-driven caspase-1 activation and intracellular IL-1β 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-1β release by each of these inflammasomes (Fig. S6 A–C). Nigericin-induced Nlpr3 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-1β continued unabated in the presence of the microtubule polymerization inhibitors nocodazole, ABT-751, CA4P, and CYT997 (Fig. S7). In conclusion, polymerized tubulin selectively and...
Mutations in 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 microtubules 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 healthy donors. To test this hypothesis, we collected PBMCs from seven healthy controls and two FMF patients with confirmed MEVF mutations in the C-terminal B30.2 domain [Table 1], patients FMF1 (M694V/R761H) and FMF2 (M694I/M694I). We did not detect secreted IL-1β 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-1β in wild-type and FMF PBMCs (Fig. 2A). FMF PBMCs that had been intoxicated with TcdA also secreted normal levels of IL-1β, a response that was efficiently blocked by the caspase-1 inhibitor Ac-YVAD-cmk (Fig. 2B). Moreover, IL-1β 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 Nlpr3 inflammasome activation (33, 34). Remarkably, however, although colchicine pretreatment abolished TcdA-induced IL-1β secretion from PBMCs of healthy individuals (Fig. 2D), it augmented the TcdA-induced IL-1β secretion from FMF PBMCs of healthy donors (Fig. 2D). The microtubule assembly inhibitors nocodazole, ABT-751, CA4P, and CYT997 also had opposite influences on TcdA-induced IL-1β secretion from FMF PBMCs and from PBMCs of healthy donors (Fig. 2E). These results further and to test whether resistance to colchicine inhibition was a defining feature of FMF patients (Fig. 2F), PBMCs that had been intoxicated with TcdA also secreted IL-1β from PBMCs of healthy donors (Fig. 2F). PBMCs from healthy donors were pretreated with colchicine before stimulation with TcdA and then were immunoblotted to detect phosphorylated Pyrin (S241), IL-1β, 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 H) and automated quantification (I and J). Immunoblot and confocal images are representative of at least three independent experiments. ns, non-significant; ***p < 0.001; ****p < 0.0001.
patients with a variety of defined MEVF 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 polyarticular; n = 7) were tested also. As with PBMCs from healthy donors, colchicine blocked TcdA-induced IL-1β 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-1β 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.

**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 unhamperepd 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-related 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).
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 Pyrin 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 Pyrin activation insensitive to microtubule manipulations by such pathogens. Given the key role of inflammasomes in antimicrobial host defense (43), the ability to engage the Pyrin 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 Pyrin 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.

**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*.

**ACKNOWLEDGMENTS.** We thank the patients and their families who provided specimens for this study; Vishwa Dixot and Nobuhiko Kayagaki (Genentech) for generously supplying mutant mice; and Amelie Fossoul (VUB-University of Ghent) and the VIB Bio Imaging Core for technical support. F.V.H., L.V.W., and N.V.O. are postdoctoral fellows with the Fund for Scientific Research-Flanders. This work was supported by Ghent University Concerted Research Actions Grant BOF14/GOA/013, European Research Council Grant 281600, and a Ballot Latour Medical Research Grant (to M.L.).

41. The Eurofever Registry and a literature review.