Pediatric RESEARCH

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BASIC SCIENCE ARTICLE

The pyrin inflammasome aggravates inflammatory cell migration in patients with familial Mediterranean fever

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BACKGROUND: Familial Mediterranean fever (FMF) is an autoinflammatory disease caused by pathogenic variants of the *MEFV* gene, which encodes pyrin. Leukocyte migration to serosal sites is a key event during inflammation in FMF. The pyrin inflammasome is a multiprotein complex involved in inflammation. Here, we aimed to determine the relationship between inflammatory cell migration and the pyrin inflammasome in FMF patients.

METHODS: Monocytes were isolated from blood samples collected from patients with FMF, healthy controls, and a patient with cryopyrin-associated periodic syndrome (CAPS), which served as a disease control. Inflammasome proteins were analyzed under inflammasome activation and inhibition by western blotting. Cell migration assays were performed with the isolated primary monocytes as well as THP-1 monocytes and THP-1-derived macrophages.

RESULTS: When the pyrin inflammasome was suppressed, migration of monocytes from FMF patients was significantly decreased compared to the migration of monocytes from the CAPS patient and healthy controls. Cell line experiments showed a relationship between pyrin inflammasome activation and cell migration.

CONCLUSIONS: These findings suggest that the increased cell migration in FMF is due to the presence of more active pyrin inflammasome. This study contributes to our understanding of the role of pyrin in inflammatory cell migration through inflammasome formation.

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IMPACT:

- The pyrin inflammasome may play a role in inflammatory cell migration.
- FMF patients show a pyrin inflammasome-dependent increase in inflammatory cell migration.
- Correlations between the pyrin inflammasome and cell migration were observed in both THP-1 monocytes and THP-1-derived macrophages.

INTRODUCTION

Autoinflammatory diseases are characterized by recurrent inflammatory attacks due to dysfunction of the innate immune system.[1] Familial Mediterranean fever (FMF), which is the most common autoinflammatory disease, is caused by altered pyrin production due to disease-causing variants in the *MEFV* gene.[2] Pyrin establishes protein–protein interactions with cytoskeletal elements, especially actin and actin-binding proteins, suggesting that it may play an important role in inflammatory cell migration.[3] In autoinflammatory diseases, immune cells, primarily neutrophils and monocytes, migrate to inflammatory sites, and in FMF, these cells migrate to serosal sites.[4] Studies have shown that pyrin is involved in regulating the cytoskeleton, and one study showed that it colocalizes with polymerized actin during cell migration and regulates the cellular distribution of PSTPIP1, a key element of the cytoskeleton.[5] In addition, it was previously reported that neutrophils from FMF patients show increased migration compared to neutrophils from healthy controls, which supports the role of pyrin in inflammatory cell migration.[6]

Pyrin has been shown to play various roles in many cellular mechanisms related to inflammatory responses. Recent studies have shown that pyrin actively participates in the structure of the pyrin inflammasome and promotes caspase-1 activation, leading to the production of interleukin-1β (IL-1β); pyrin inflammasomes also play a role in pyroptosis.[7,8] The mechanisms underlying pyrin inflammasome activation have been explored in several studies. Magnotti et al.[9] found that pyrin dephosphorylation was sufficient for inflammasome activation in patients with FMF, whereas a second control mechanism was needed for inflammasome activation in normal controls and non-FMF patients. In one study, *MEFV* expression and pyrin inflammasome activation were increased following inhibition of the mTOR pathway by receptor-interacting protein kinase 3.[10] Sharma et al.[11] found that the contribution of pyrin to inflammation and the maturation of IL-18, a crucial component for promoting barrier integrity, is important for preventing colon inflammation and tumor formation.

Most studies investigating the effect of inflammasome activation on cell migration have mainly focused on the NLRP3

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(NOD-, LRR- and pyrin domain-containing protein 3) inflammasome. In an animal model of multiple sclerosis, the NLRP3 inflammasome was shown to play a role in inducing T helper cell migration.[12] In addition, the NLRP3 inflammasome was found to play a role in regulating the proliferation and migration of A549 lung cancer cells.[13] However, to date, no study has investigated the role of the pyrin inflammasome in cell migration.

This study aimed to understand whether the activity of the pyrin inflammasome is associated with the increased inflammatory cell migration seen in FMF patients. Here, we report that excess pyrin inflammasome activation increases cell migration in patients with FMF.

METHODS

Patient groups

Eleven patients who visited the Hacettepe University Pediatric Rheumatology Department and were diagnosed with FMF according to PRINTO/Eurofever score and Yalcinkaya-Ozen criteria were selected as the study group. All FMF patients had M694V pathogenic variants in both alleles of the *MEFV* gene. One patient with cryopyrin-associated periodic syndrome (CAPS), who had pathogenic variants (Y570F/Y570F) in the *NLRP3* gene, and seven control individuals without any autoinflammatory disease, all aged 5–18 years, were also included in the study.[14,15] Each subject and his/her parents signed a free informed consent form.

Peripheral blood mononuclear cell (PBMC) isolation

Blood samples were collected from the patients diagnosed with FMF or CAPS and the healthy individuals into 10 mL heparinized tubes and were delivered to the laboratory at room temperature. Each blood sample was transferred to a 50 mL tube, and then 10 mL of phosphate-buffered saline (PBS) and an equal volume of lymphocyte separation medium were added. The samples were centrifuged at $700 \times g$ for 20 min at room temperature to separate the cells into layers. PBS was added to collect the PBMC layer, which was transferred to a new 50 mL tube. The volume was brought to 50 mL with PBS. Then, the mixture was centrifuged at $700 \times g$ for 7 min at room temperature. The cell pellet was dissolved in Opti-MEM. For monocyte culture, the isolated PBMCs were suspended in serum-reduced cell medium and incubated in 12-well cell culture plates for 2 h at 37 °C in a 5% CO_2 incubator for monocyte enrichment.[16]

Activation and suppression of the inflammasome

To induce monocyte activation, the cells were plated in 12-well plates containing 1 µg/mL lipopolysaccharide (LPS) in Opti-MEM (Invitrogen) and incubated for 6 h. To inhibit inflammasome formation, the primary monocytes were treated with LPS (1 µg/mL) and arachidonic acid (AA) (60 µM). Opti-MEM alone was used as a control. Cells were incubated with LPS and AA at the determined doses for 6 h, and then harvested in 35–40 µL of protein lysis solution to isolate proteins for western blotting. In addition to the primary monocyte experiment, LPS and *Clostridium difficile* toxin B (TcdB), a pyrin inflammasome activator, were used to activate the pyrin inflammasome in THP-1 monocytes and THP-1-derived macrophages.

Protein isolation and immunoblotting

Protein was isolated from cell suspensions stored in Triton X-100-containing protein lysis buffer using the heat-shock method. Protein was isolated from the cell culture medium using the methanol–chloroform method. The concentration of the protein isolated from the cells and culture supernatant was determined using the BCA Protein Assay kit. Equal protein samples were loaded and separated by electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated with anti-ASC (Cell Signaling Technology), anti-pro-IL-1β (Cell Signaling Technology), and anti-pyrin (a kind gift from J.J. Chae, NIH) antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected with anti-GAPDH (Sigma) antibodies as an equal loading control. Equal protein samples from cell culture were loaded and separated by electrophoresis and then transferred to nitrocellulose membranes. The membrane was incubated with anti-cleaved IL-1β antibodies (Cell Signaling Technology) to detect secreted IL-1β. The Coomassie blue-stained gel after transfer was used as a control for equal loading.

Transwell cell migration assay

A total of 1.5×10^4 cells in 400 µL of Opti-MEM were seeded into the upper compartment of a Transwell chamber. Then, 600 µL of Opti-MEM containing 10% fetal bovine serum (FBS) was placed in the lower chamber, and the cells were allowed to migrate for 2.5 h at 37 °C and 5% CO_2. The migrated cells were then treated with 1 mM calcein-AM and incubated for 30 min at 37 °C in a 5% CO_2 incubator. The upper chamber was photographed at ×4 magnification using a Leica IM50 fluorescence microscope. Cells that migrated to the lower chamber were visualized by calcein-AM staining using a Leica IM50 fluorescent microscope, and photographs of each well were taken using Leica Application Suite 3.1 software at ×4 magnification. Similar experiments were performed with undifferentiated THP-1 cells.

Wound healing assay

In wound healing experiments, 2×10^6 THP-1 cells were seeded into a 6-well plate and incubated for 48 h with 200 ng/mL phorbol-12-myristate-13-acetate (PMA) to induce macrophage differentiation. Wounds were formed using a 10 µL pipette tip. The wounds were incubated for 0, 6, and 24 h, and then imaged at ×4 magnification to assess healing.

Statistical analysis

Cells that migrated to the lower chamber of the Transwell apparatus were quantitated using the "particle analysis" method in ImageJ 1.46 software. The gap at each time interval and the closure percentage were calculated as the cells migrated over time using ImageJ 1.46 software. Protein expression levels and wound closure were determined using GraphPad Prism 6.1, which was also used to draw graphics and perform the statistical analysis between groups. Differences were determined using the Mann–Whitney *U* test and Student's *t* test. Statistical significance was set at $p < 0.05$.

RESULTS

Clinical outcomes of study groups

A total of 11 patients with FMF (six females and five males, median age 11.7 years (4–19)), one patient with CAPS (male, 10 years), and seven healthy children (three females and four males, median age 13 years (5–18)) were enrolled in this study. All FMF patients were homozygous for M694V, and the CAPS patient had somatic NLRP3 mosaicism (Y570F/Y570F). All patients with FMF were treated with colchicine. Four of them were colchicine resistant and were treated with anti-IL-1 antibodies. The CAPS patient was treated with anti-IL-1 antibodies and on-demand steroids. The demographic and clinical characteristics of the patients are summarized in Table 1.

Levels of inflammasome proteins under different conditions

As expected, ASC and pro-IL-1β protein levels in cell lysates and cleaved IL-1β protein levels in cell culture medium were increased under LPS stimulation and decreased following inflammasome inhibition with AA. There were no significant differences in the expression levels of pyrin protein between FMF patients and control subjects. Therefore, the difference in pyrin function between the groups is due to an increase in the rate of participation in the

inflammasome structure rather than an increase in protein expression. Under the same conditions, it was found that the inhibition of other inflammasome proteins was lower in patients with FMF (Fig. 1).

Migration patterns of inflammatory cells from FMF/CAPS patients and controls

The application of LPS significantly increased cell migration, while the application of both AA and LPS significantly decreased cell migration. Although the migration of cells from FMF patients increased significantly after LPS application, cell migration was decreased to less than that of the control when both AA and LPS were applied (Fig. 2a, b). The migration of cells from the CAPS patient was significantly increased following the application of LPS, whereas cell migration decreased similar to that of the control when AA and LPS were co-applied (Fig. 2c).

Table 1. Demographics and clinic data of patients, *n* (%) or mean (minimum–maximum).

	Total ($n = 19$)	FMF ($n = 11$)	CAPS ($n = 1$)	Control ($n = 7$)
Age (year)	12.1 (4–19)	11.7 (4–19)	10	13 (5–18)
Female	9 (47.4)	6 (54.5)	–	3 (42,9)
Male	10 (52.6)	5 (45.5)	1	4 (57.1)
Body mass (kg)	40.2 (15–66)	35.5 (15–59)	20,5	50.6 (21–66)
Diagnosis age	4.1 (1–10)	4.2 (1–10)	3	–
MEFV (M694V/M694V)	11	11	–	–
NLRP3 (Y570F/Y570F)	1	–	1	–
Colchicine	11	11	–	–
Canakimunab	4	3	1	–
Anakinra	1	1	–	–
Other drugs[a]	2	1	1	–
CRP (mg/dL)	0.66 (0.1–1.8)	0.96 (0.1–1.8)	0.8	0.157 (0.1–0.3)
Sedimentation	10.5 (2–53)	15.7 (2–53)	8	2.7 (2–4)
AIDAI Scores	–	5.67 (0–15)	21	–

CAPS cryopyrin-associated periodic syndrome, *CRP* C-reactive protein, *F* female, *FMF* familial Mediterranean fever, *M* male, *MEFV* MEditerranean FeVer gene, *NLRP3* NOD-, LRR- and pyrin domain-containing protein 3 gene.
[a]Methotrexate and corticosteroid.

When we evaluated our study groups together under the same conditions, the inhibition of inflammasome activation, as shown by the release of IL-1β into serum, was lower in cells derived from FMF patients than in the cells from the controls (Fig. 3a). The inhibition ratio of the migration of cells from FMF patients was significantly different from that of cells from the CAPS patient and control individuals (Fig. 3b). Overall, IL-1β release and inflammatory cell migration were higher in patients with FMF.

Effect of the pyrin inflammasome on the migration of THP-1 cells and THP-1-derived macrophage cells

The migration of THP-1 monocytes increased following pyrin inflammasome activation (LPS + TcdB) and decreased following inflammasome inhibition (LPS + TcdB+AA; Fig. 4). In wound healing experiments using THP-1-derived macrophages, the rate of wound closure increased with pyrin inflammasome activation (LPS + TcdB) and decreased with inflammasome suppression (LPS + TcdB+AA) at 6 and 24 h after treatment (Fig. 5). These results suggest that pyrin inflammasome activation triggers inflammatory cell migration.

DISCUSSION

Inflammasome activation is a major phenomenon that occurs in innate inflammatory cells in response to endogenous and exogenous stimuli. When inflammasomes are activated, various biological processes in the cell, such as pyroptosis, migration, etc., can be induced along with cleavage of IL-1β. Inflammasomopathies, a subtype of systemic autoinflammatory diseases, are caused by the dysregulation of inflammasome activation.[17] FMF is also considered to be an inflammasomopathy, as this disease affects the pyrin inflammasome.[8] Pyrin plays a role in cytoskeleton organization through its interaction with key components of the cytoskeleton, such as actin and PSTPIP1 among others.[5,18] Waite et al.[3] found that pyrin and ASC localize in dynamically polymerizing actin-rich tails and pyrin binds to the actin-binding proteins VASP and Arp3. Regarding the function of pyrin protein in neutrophils, Balci-Peynircioglu et al.[6] showed that pyrin-silenced HL60 cells could not be polarized, and they were not able to migrate properly. In the present study, we demonstrated the changes in inflammatory cell migration that occurred following inflammasome activation and inhibition.

Primary monocytes are important cells that are useful for studying the migration potential of immune cells following inflammasome inhibition/activation. Due to the reduced migration capacity of cryopreserved cells, which can cause deviations in the results, freshly isolated primary monocytes were used in this

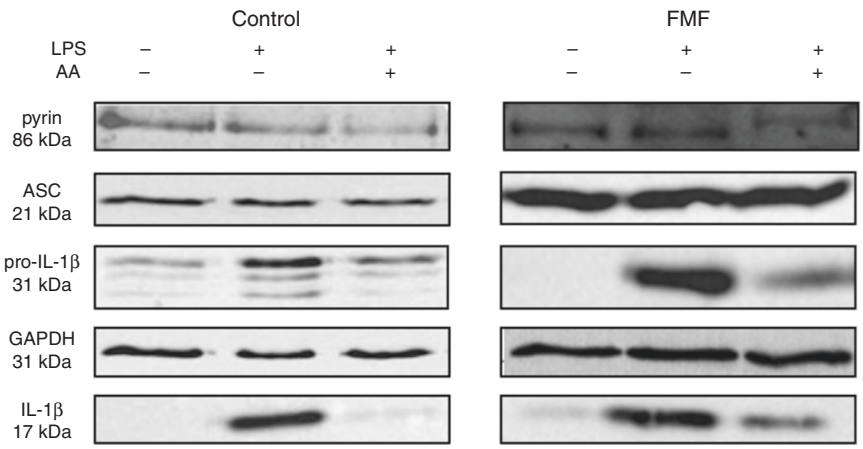

Fig. 1 Expression analysis of inflammasome proteins. Western blot images showing the amount of various proteins in cell lysates (pyrin, ASC, and pro-IL-1β) and cell culture medium (IL-1β) in immune cells from both healthy controls and patients with FMF under different conditions. GAPDH was used as an equal loading control.

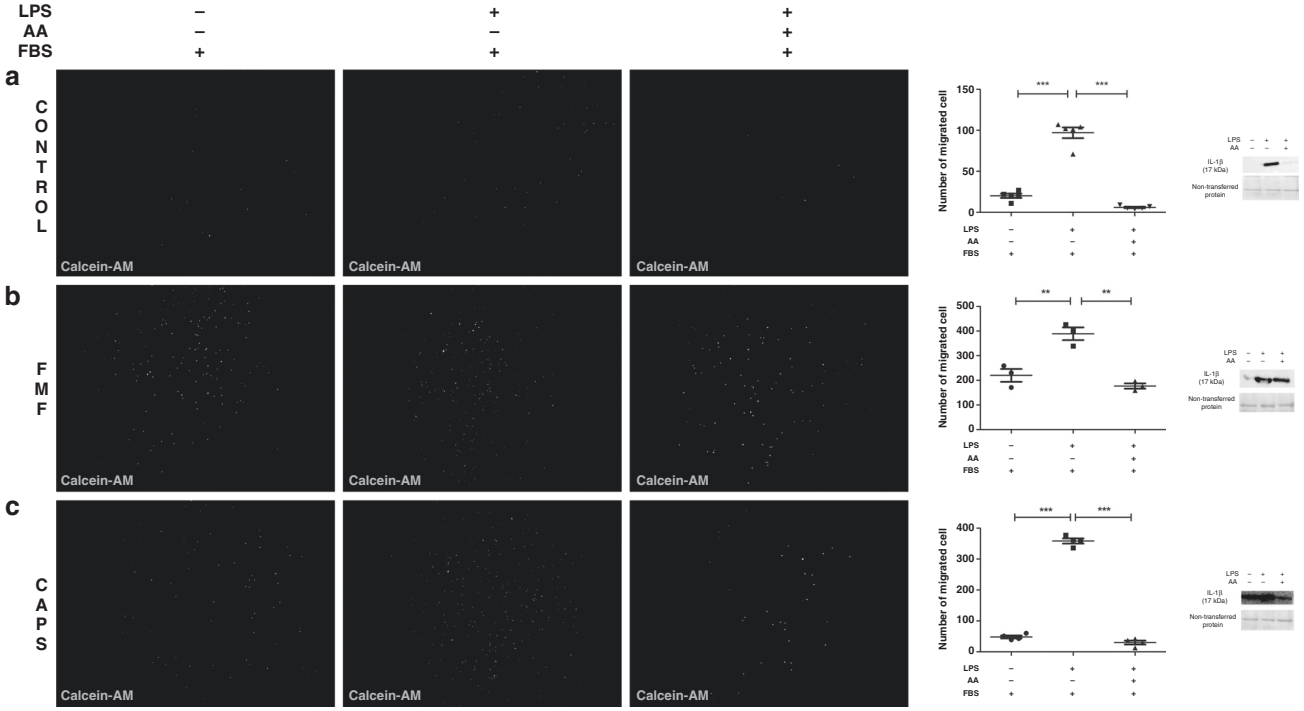

Fig. 2 Determination of monocyte migration and IL-1β secretion. Calcein-AM fluorescence-stained image of immune cells from **a** control individuals, **b** patients with FMF, **c** a CAPS patient migrating to the lower compartment of a Transwell apparatus containing 10% FBS as a chemoattractant. Graphs showing the number of cells migrating to the lower compartment under different conditions. Western blot of the mature IL-1β released into the cell culture medium under different conditions. Non-transferred protein was used as an equal loading control. $^{**}p < 0.01$, and $^{***}p < 0.001$.

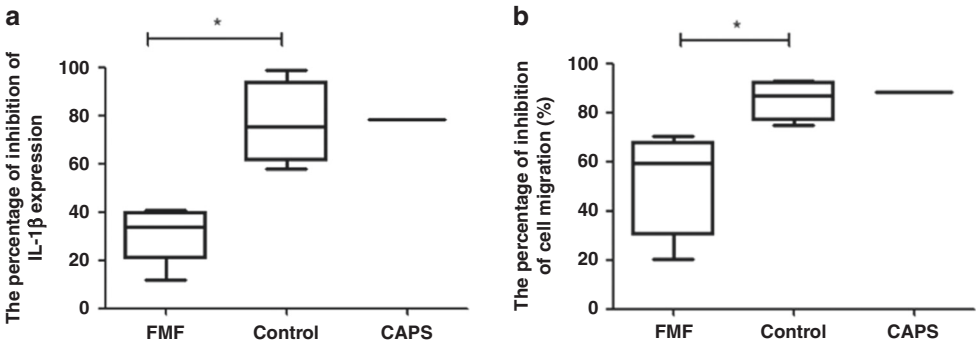

Fig. 3 Comparison of inhibition of cell migration and IL-1β secretion in study group. a Graph showing the suppression rates of mature IL-1β expression following inhibition of the inflammasome of cells from patients with FMF, healthy controls, and a CAPS patient. **b** Graph showing the migration suppression rates of cells obtained from patients with FMF, healthy controls, and a CAPS patient following inflammasome inhibition. $^{*}P < 0.05$.

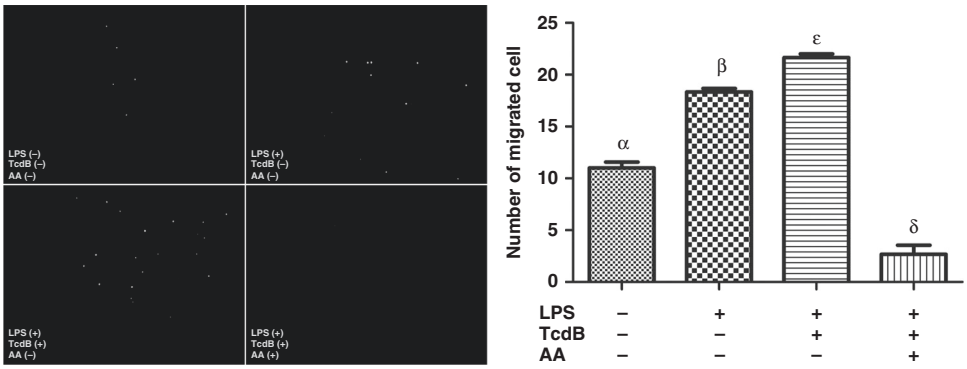

Fig. 4 Transwell experiments performed in THP-1 cells. Image of the calcein-AM fluorescence-stained THP-1 cells migrating to the lower compartment of a Transwell apparatus containing 10% FBS as a chemoattractant. Graphs showing the number of migrating cells under different conditions. α vs. δ and β vs. ε; $p < 0.01$, α vs. β, α vs. ε, ε vs. δ, β vs. δ; $p < 0.001$.

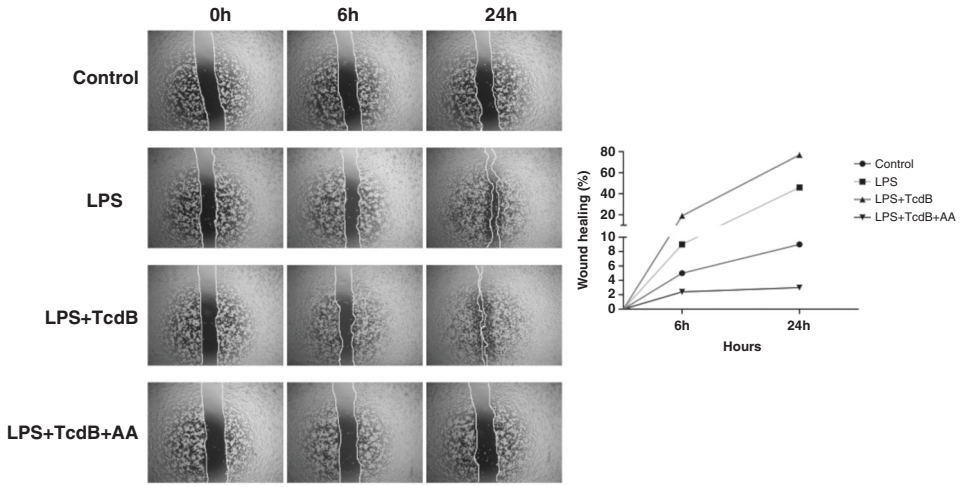

Fig. 5 Wound healing assay performed in THP-1-derived macrophages. Closure of the wound in THP-1-derived macrophages at 6 and 24 h after LPS, LPS + TcdB, or LPS + TcdB + AA treatment.

study. Inflammasome activation occurs in two steps: priming and activation. Although priming is mostly triggered by LPS, there are many different activators for the various inflammasomes. In this study, primary monocytes from patients with FMF were stimulated with LPS, which initiates priming. In the primary monocyte experiments, we skipped the activation step because sufficient supernatant IL-1β cleavage was obtained, and we were interested in investigating the effect of basal inflammasome activation on inflammatory cell migration. Therefore, a significant effect was observed with only LPS stimulation. It has been shown that in patients with FMF, cell migration is increased following activation of the pyrin inflammasome. CAPS is another autoinflammatory disease characterized by excessive activation of the NLRP3 inflammasome. The migration pattern of cells from the CAPS patient was similar to that of cells from the control individuals, strengthening the hypothesis that increased cell migration may be specific to FMF. The findings obtained using primary cells from patients and healthy controls were validated using both THP-1 monocytes and THP-1-derived macrophages, and similar results were obtained under specific pyrin inflammasome activation/inhibition conditions. These results support our hypothesis regarding the importance of the pyrin inflammasome in cell migration. Indeed, this study showed that FMF patients have a low threshold for pyrin inflammasome activation, which is consistent with the literature. We also reported a lower inflammasome suppression rate with the application of the inhibitory molecule AA for primary monocytes from FMF patients than for monocytes from both the CAPS patient and healthy controls.

The most commonly used approach in the treatment of FMF is the daily administration of colchicine, a tubulin-depolymerizing agent. However, a small percentage of patients, who are referred to as colchicine resistant, do not respond to colchicine.[19,20] Colchicine has been reported to reduce ASC specks, the distribution of PSTPIP1, pyrin-interacting proteins, and inhibit cell migration.[21] Although all the FMF patients in our study group received daily colchicine (Table 1), they had a higher cell migration rate under both basal and activation conditions.

Studies have shown that, in addition to genetic factors, epigenetic regulators, such as microRNAs, can affect various cellular processes, such as migration, in most rheumatic diseases.[22,23] Moreover, products derived from the gut microbiota can mediate immune cell migration and trigger systemic autoinflammatory/autoimmune diseases.[24,25] Hence, they may also have effects on major differential mechanisms, such as cell migration, in FMF and should be investigated.

This study has some limitations. First, we were only able to include one CAPS patient who met the criteria of the study group since CAPS is a very rare autoinflammatory disease. The second limitation of the study is that the sampling was performed at a single time point, but we observed a difference in the cell migration rate at different time points. Despite these limitations, the results of our study, along with relevant literature, suggest that the pyrin inflammasome is more active in FMF disease, and the increased cell migration seen in patients with FMF may be pyrin inflammasome dependent.

CONCLUSIONS

The pyrin inflammasome modulates the migration of monocytes, and excess activation of the pyrin inflammasome due to the presence of mutant pyrin causes aberrant cell migration in patients with FMF. We suggest that the relationship between the pyrin inflammasome and cell migration may be important for other diseases that affect the pyrin inflammasome.

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AUTHOR CONTRIBUTIONS

T.H.A., Z.Y.A.-U., and B.B.-P.: conceptualization; T.H.A.: investigation; T.H.A., S.D., and S.O.: resources; T.H.A.: writing the original draft; B.B.-P. and S.O.: writing the review and Editing; T.H.A., Z.Y.A.-U., and B.B.-P.: funding acquisition. All authors have read and approved the final manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Statement of consents: The study was approved by the Hacettepe University Non-interventional Clinical Researches Ethics Board (GO 17/514). Written consent was obtained from all parents and children.

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