Potential role of pyrin, the protein mutated in familial Mediterranean fever, during inflammatory cell migration

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ABSTRACT

Familial Mediterranean fever (FMF), the most common of the systemic autoinflammatory disorders, is caused by mutations in the MEFV *(Mediterranean Fever) gene, which encodes the protein pyrin. Neutrophils, one of the major components during inflammation, are the main cell type that expresses pyrin. In response to an inflammatory stimulus, neutrophils migration to their main active site. To date, several pyrin-interacting proteins have been demonstrated to co-localise with the cytoskeletal protein actin, which is important in the process of neutrophil migration and raises the question of whether pyrin plays a role in the actin cytoskeletal network during inflammatory cell migration. In this study, we examined the possible role of pyrin during inflammatory cell migration in neutrophils. We generated a cell migration assay with neutrophils and primary neutrophils from patients. We also knocked down pyrin expression using siRNA and then performed cell migration assay. We showed co-localisation of pyrin and F-actin at the leading edge during inflammatory cell migration. In pyrin knocked down cells, we identified a significant decrease in neutrophil migration. In addition, we demonstrated a dramatic increase in migration in the neutrophils of FMF patients compared with a healthy control group. These data together provide new insight into the cellular function of pyrin and demonstrate an important link between pyrin and polymerising actin in the process of inflammatory cell migration.*

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Introduction

Systemic autoinflammatory disorders are explained as heritable human diseases characterised by fever and inflammation in the absence of T- or B-cell involvement (1). Familial Mediterranean fever (FMF), the most common of these autoinflammatory disorders, is characterised by sporadic and self-limited attacks of fever with painful inflammation caused by mutations in the *MEFV* (Mediterranean Fever) locus (2, 3). The inflammatory attacks involve massive neutrophil influx to affected sites, such as the abdomen, chest or joint. FMF is prevalent mainly in the Mediterranean region, and the frequency of several of the known *MEFV* mutant alleles is high in non-Ashkenazi Jews, Armenians, Arabs, and Turks (4). However, it is also observed worldwide due to the increase in the rate of population movements in this century (5, 6). To date, 134 mutations have been identified in the *MEFV* gene (INFEVERS database, 2018), of which M694V, M680I, E148Q, V726A were reported as the most common ones in different populations (7, 8). In populations where FMF is highly prevalent, a proportion of patients with no MEFV mutations may carry causative mutations in other genes related with other systemic autoinflammatory diseases and the clinical findings may not be explained with the phenotype expected of the mutation identified (9).

Although phenotype-genotype correlations in FMF have not been explained, several studies have demonstrated that it appears to be a more severe disease in patients with M694V mutation (4, 8, 10). Prophylactic treatment with colchicine, a known microtubule toxin, lessens the number and intensity of attacks.

Pyrin, the protein product of the *MEFV* gene, has been shown to be a major component of inflammasome, dependent on the adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD) which leads to an increase in caspase-1 activation and IL-1β processing. In 2014, Xu *et al.*

have shown that the activation of pyrin inflammasome is taking place as a result of inactivation of Rho GTPases by certain bacterial toxins (11, 12).

Pyrin is expressed mainly in neutrophils, eosinophils, cytokine-activated monocytes and some fibroblasts (13). Neutrophils are one of the major components of the innate immune system, which rapidly respond to danger signals and migration to their main active site following an inflammatory stimulus. The main mechanisms of neutrophils include phagocytosis, respiratory burst, the formation of neutrophil extracellular traps (NETs), and the production of cytolytic enzymes, cytokines and chemokines (14). Cell movement in general requires highly organised cytoskeletal rearrangements that lead to directed migration (15). As a response to various sources, such as formulated peptides, products of the complement cascade, and chemokines, the increase in the polymerisation of G to F-actin is important and one of the early key events in the process of neutrophil migration toward the region of inflammation (15, 16). Pseudopod extension due to polarity formation in neutrophils occurs through localised polymerisation of F-actin at the plasma membrane of cells exposed to the highest chemoattractant concentration (15). To date, several actin-binding proteins, such as the Arp2/3 complex, filamin B, cortactin, α actinin-4, and cofilin, have been demonstrated to play a role in neutrophil migration (17, 18).

In previous studies, Waite *et al*. demonstrated that pyrin, the protein product of the *MEFV* gene, and ASC (Apoptosis-associated Speck protein with CARD domain), a known pyrin-interacting protein, were concentrated in dynamically polymerising actin-rich tails generated by *Listeria monocytogenes*, and pyrin was shown to bind to actin-binding proteins VASP and Arp3 (19). More recent work has indicated that pyrin and another pyrin-interacting protein, PSTPIP1, the protein that is mutated in PAPA syndrome (Pyogenic Arthritis, Pyoderma gangrenosum, and Acne), an autoinflammatory disease, co-localise with actin at the leading edge of migrating cells in a cell line model (20). Additional data showed that shorter filopodia exist in colchicine-treated monocyte cell lines, possibly due to the effect of colchicine causing actin re-organisation while the microtubule structure is intact (21). These studies concerning the interaction of pyrin with acting-binding proteins and related to cell migration process support the idea that pyrin may play a role in the actin cytoskeletal network during inflammatory cell migration.

Here, we examined more closely the possible role of pyrin during inflammatory cell migration in both neutrophils-like cell line models and primary human neutrophils. We generated a cell migration assay with neutrophil-like HL- 60 cells and human neutrophils and showed co-localisation of pyrin and F-actin at the leading edge during inflammatory cell migration. We knocked down the expression of pyrin using siRNA and identified a decrease in both cellular polarisation and the cell migration ratio. In addition, we demonstrated dramatic differences in inflammatory cell migration in the neutrophils of FMF patients with different mutations compared with that of the healthy control group. Together, these data could provide a previously unrecognised means to connect the pyrin-actin relationship in the cytoskeletal network to inflammatory cell migration.

Methods

Cell isolation, culture and differentiation (cell lines and primary cells)

The neutrophil-like cell line HL-60 (American Type Culture Collection, Manassas, VA, USA) was cultured and differentiated according to a previously described protocol (22). Briefly, cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Eugene, OR, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (v/v) and 1% penicillin/streptomycin (v/v) at 37°C in 5% CO_2 in non-coated T75 flasks. These cells were then plated in the CC2, Nunc Lab-Tek1 II Chamber SlideTM system (Milian Dutscher Group, Switzerland) for cellular polarisation and immunostaining experiments. HL-60 cells were cultured in culture medium with 1.28% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) for 6 days for differentiation.

Human neutrophils were isolated from peripheral blood samples (~10 ml) taken from 20 healthy donors and 27 FMF patients. Age matched healthy controls were used for each experimental set-up. The median ages were 10 for patients, and 11 for healthy control individuals. The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, attack frequency (number of attacks during the previous year), and disease state (attack or attack-free period) in FMF patients were assessed using the blood drawn for experiments.

Hacettepe University, Ethics Committee, approved this study (Ethical Approval: GO15/90). All blood samples from healthy donors and patients who participated in this project were obtained after informed consent had been provided according to guidelines by the local ethics committee. Written consent was obtained from patients, since our study included minors, we obtained consent from parents. Anticoagulant blood was layered onto Lympholyte-poly solution (Cederlane Laboratories, Cat. No: CL5071) (1:1 vol with blood). After the first centrifugation, at 500 g for 35 minutes, 2 distinct bands as mononuclear cells (upper band) and polymorphonuclear cells (lower band) were observed. The high-density neutrophil layer was collected from the lower pellet fraction and was centrifuged at 350 g for 10 minutes. Next, the neutrophils were suspended in 6-7 ml of lysis buffer to remove contaminating erythrocytes. After centrifugation at 250 g for 5 minutes, the final neutrophil pellet was suspended in 100 μl of PBS for flow cytometry analysis and cytospin, or in RPMI-1640 media, containing 2 mM L-glutamine, 100 U/ml penicillin (1%), 100 μg/ml streptomycin (1%) and FBS (10%) (Sigma-Aldrich) for immunocytochemistry and chemotaxis assays. Neutrophil purity was measured by MayGrünwald-Giemsa staining (Sigma-Aldrich) and flow cytometry (BD FACS Aria II).

siRNA transfection

HL-60 cells were transiently transfected by Lipofectamine RNAiMAX Reagent (Invitrogen) using 14 pmol of Silencer Select MEFV siRNA, s8660 (Ambion). For the control group, cells were transfected with 14 pmol of Select Negative Control siRNA #1 (Ambion). We used scrambled siRNA transfected cells and non-transfected cells as control for these experiments (Supplementary Fig. 1). After 24 hours of transfection, the cells were incubated with differentiation medium for 6 days. Transfection was repeated at the 4th day of the differentiation. The cells were then harvested for protein isolation and detection of the pyrin protein level.

Protein extraction

HL-60 cells were lysed in Triton X-100 lysis buffer (10 mM Tris–base at pH 7.4, 300 mM NaCl, 2 mM EDTA and 0.5% Triton X-100 with protease inhibitor cocktail (Complete Mini, EDTA-free protease inhibitor cocktail tablets; Roche)) and centrifuged at 13,000 g for 30 minutes at 4°C. The concentrations of the total protein extracts were measured using a BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cell lysates were stored at -80°C until Western blot analysis.

Western blotting

Approximately 40 μg of the total protein extracted from HL-60 cells was separated by 12% SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis and was transferred to a nitrocellulose membrane. The membranes were incubated for 1 hour with the mouse polyclonal anti-pyrin antibody after blocking in 5% non-fat dry milk/0.01% Tween 20 in TBS (Tris- buffered saline). The housekeeping gene product of mouse monoclonal anti-GAPDH was used as a loading control. Next, horseradish peroxidase-conjugated secondary antibodies were used, followed by SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) detection.

Cellular polarisation

HL-60 cells were stimulated for migration with 100 nM N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (Sigma-Aldrich) for 24 hours at 37°C in 5% CO_2.

Following isolation, human neutrophils (4×10^5/500 μL in RMPI-1640) were incubated for 2 hours at 37°C in 5% CO_2 for adhesion to glass-bottom slides (Nunc Lab-Tek1 II Chamber SlideTM). Next, neutrophils were treated with 50 μM fMLP for 1 hour at 37°C in 5% CO_2 to trigger polarisation.

Cell chemotaxis assay

A 24-well plate fitted with a 3-μm filter (Greiner), a suitable pore size for HL-60 cells and neutrophils, was used for the chemotaxis assay (23, 24). HL-60 cells (4×10^5 cells) and neutrophils (15×10^3/400 μL in RPMI-1640) were loaded onto the upper compartment of the filter and were triggered to migration toward the fMLP gradient (100 nM) in the lower compartment. Equal numbers of cells were loaded onto the upper compartment as a control condition in which the lower compartment contained fMLP-free medium. Cells were incubated at 37°C for 24 hours. Following incubation, the filter was removed, and the cells were stained with 1 mM Calcein-AM (Sigma-Aldrich) at 37°C for 15 minutes. Cells stained with Calcein-AM were visualised by fluorescence microscopy (×200 magnification; Leica DMIL microscope equipped with analysis software Leica Application Suite 3.1). Calcein-AM is a non-fluorescent cell permeable derivative of calcein that becomes fluorescent upon hydrolysis within the cytosol which is used as a cell viability stain and as a neutral substrate for multidrug (MDR) efflux transporters. Thus at the end of transwell assay, only live cells can be visualised, and apoptotic cells do not affect the calculation at the results. The cells migrating toward the lower compartment were counted using the 'analyse particles' function in ImageJ 1.46 software.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in PBS and then were permeabilised in PBS with 1% Triton-X-100 (Sigma-Aldrich) at room temperature. Following rinsing with PBS containing Tween 20 (Sigma-Aldrich), nonspecific staining was blocked by the combination of the Fc Receptor blocker (Innovex Biosciences) for 30 minutes and PBS w/1% BSA/3% goat serum at room temperature for 1 hour. All antibody incubations were performed at room temperature, and the cells were washed in PBS between each incubation step. Cells were treated with DAPI (1 mg/ml) for 1 minute, washed three times with PBS w/Tween 20 and analysed by fluorescence microscopy. Negative control stainings for both actin and pyrin were performed. Cells treated with DAPI, antibody and phalloidin were analysed by Carl Zeiss LSM-Pascal confocal microscope. Cells treated with calcein-AM were analysed by Leica DMIL or Carl-Zeiss Axioplan 2 equipped with an ICAM photosystem fluorescence microscope.

Antibodies

Anti-pyrin antibody (Abnova, Walnut, CA, USA) was used for immunocytochemistry and Western blotting. F-actin filaments were visualised using AlexaFluor488 Phalloidin (Molecular Probes by Invitrogen, Eugene, OR, USA). The secondary antibodies Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit were obtained from Molecular Probes by Invitrogen. Horseradish peroxidase (HRP)-conjugated immunoglobulin-G was purchased from Invitrogen-Molecular Probes (Carlsbad, CA, USA).

Statistical analysis

All the graphs were created using GraphPad Prism 6.1 Software (GraphPad Software, San Diego, CA, USA). Non-parametric Mann-Whitney U-test and Student's *t*-test were used for studies comparing differences between two groups. Differences were considered significant when $p<0.05$.

Results

Pyrin co-localises with actin at the leading edge in migrating cells

The cytoskeletal protein actin is localised under the cellular membrane, and

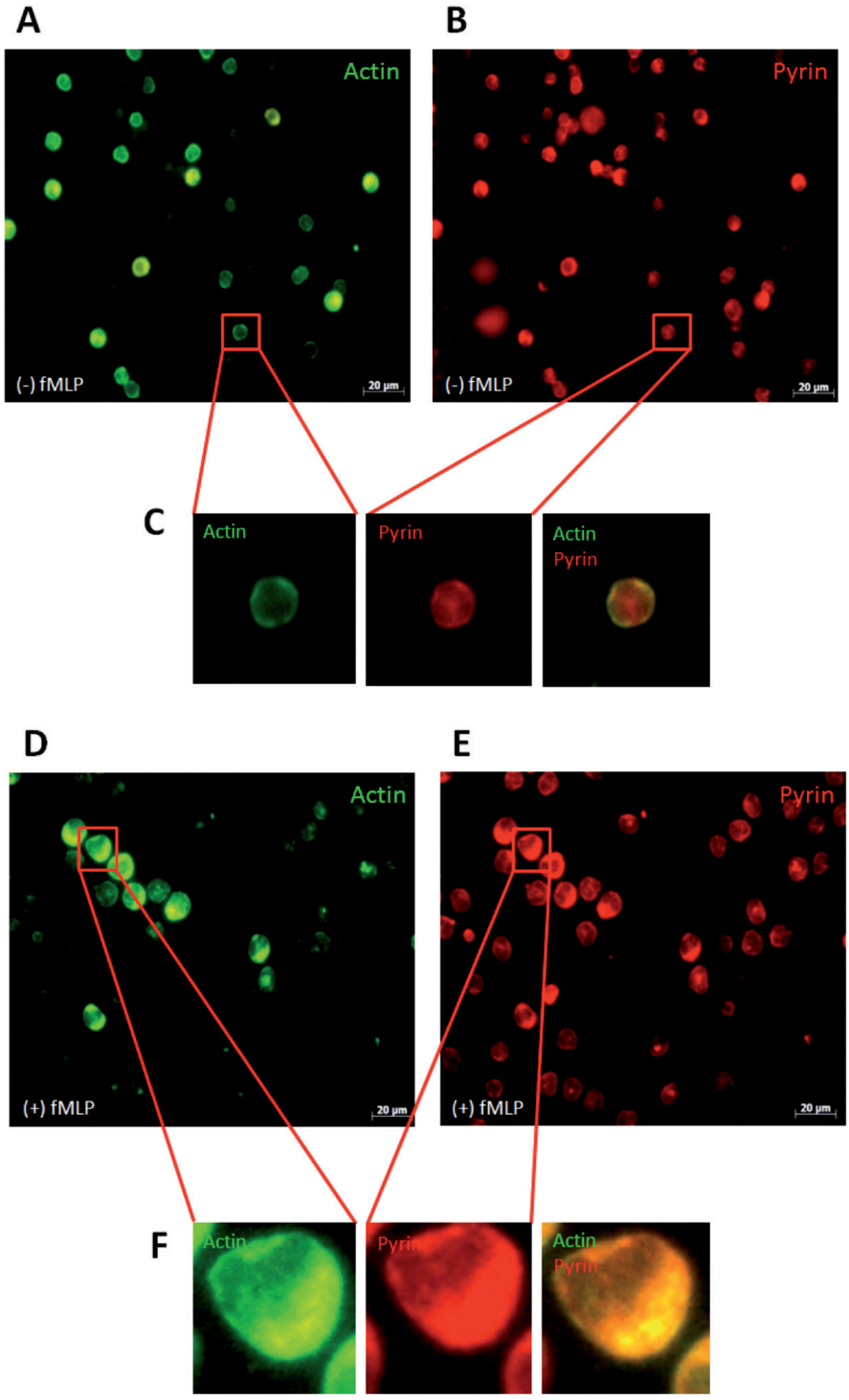

Fig. 1. Pyrin-actin co-localisation in human neutrophils. (**A-B**) Double staining of actin (green) and pyrin (red) in unstimulated primary human neutrophils. (**C**) Selected cell from A and B for zoom-in view. (**D-E**) Double staining of actin (green) and pyrin (red) in stimulated primary human neutrophils. (**F**) Selected cell from D and E for zoom-in view. Pyrin co-localises with polymerising actin at the leading edge in migrating neutrophils. Scale bar = 20 µm. Experiments were performed technically in duplicate.

F-actin appears concentrated on the cell's polarised edge after stimulation (23). Both differentiated HL-60 cells and human neutrophils exhibited the morphology of migrating cells after stimulation with fMLP, and the nucleus was pulled towards the leading edge, which was actin rich. Pyrin staining was concentrated on the actin-rich leading edge in differentiated HL-60 cells concordantly with our previous research (20) and in human neutrophils after stimulation. In the same set of cells stained with both anti-pyrin and phalloidin, we found consistent co-localisation of these proteins in human neutrophils (Fig. 1A-F). Because the leading edge is a known region for polymerising actin, and phalloidin selectively labels F-actin (24), our staining experiments supported the idea that pyrin co-localises with polymerising actin at the leading edge in migrating neutrophils.

Pyrin knockdown leads to reduction in inflammatory cell migration in neutrophils-like cells

To define the specific function of pyrin in migration, we reduced the expression of the *MEFV* gene in HL-60 cells using small interfering RNAs (siRNAs). The knockdown effect of the siRNA was then checked using Western blot analysis and quantified by ImageJ and GraphPad Prism 5 analysis programmes (Fig. 2C). Two bands indicated two isoforms of the MEFV gene, as consistent with the data obtained by Diaz *et al*. in synovial fibroblasts (25). The original uncropped and unadjusted blots are provided in supporting information (Supplementary Fig. 2). After optimising the transfection protocol, pyrin was silenced with 80% efficiency in HL-60 cells. Differentiated cells were stimulated with fMLP, and then the cellular polarisation and chemotaxis ratios were compared with those of the control group. We used scrambled siRNA transfected cells and non-transfected cells as control for these experiments. We did not observe any change in cell migration in fMLP (-) conditions and in control cells which supports the fact that the effect we see in MEFV siRNA is not due to a immunostimulatory effect of siRNA (Supplementary Fig. 1). Phalloidin and pyrin co-staining of stimulated cells showed that knockdown of *MEFV* significantly reduced the polarisation ratio in pyrin-silenced HL-60 cells. They showed less actin polymerisation than control cells and appeared as round shaped in morphology (Fig. 2A). In agreement with these

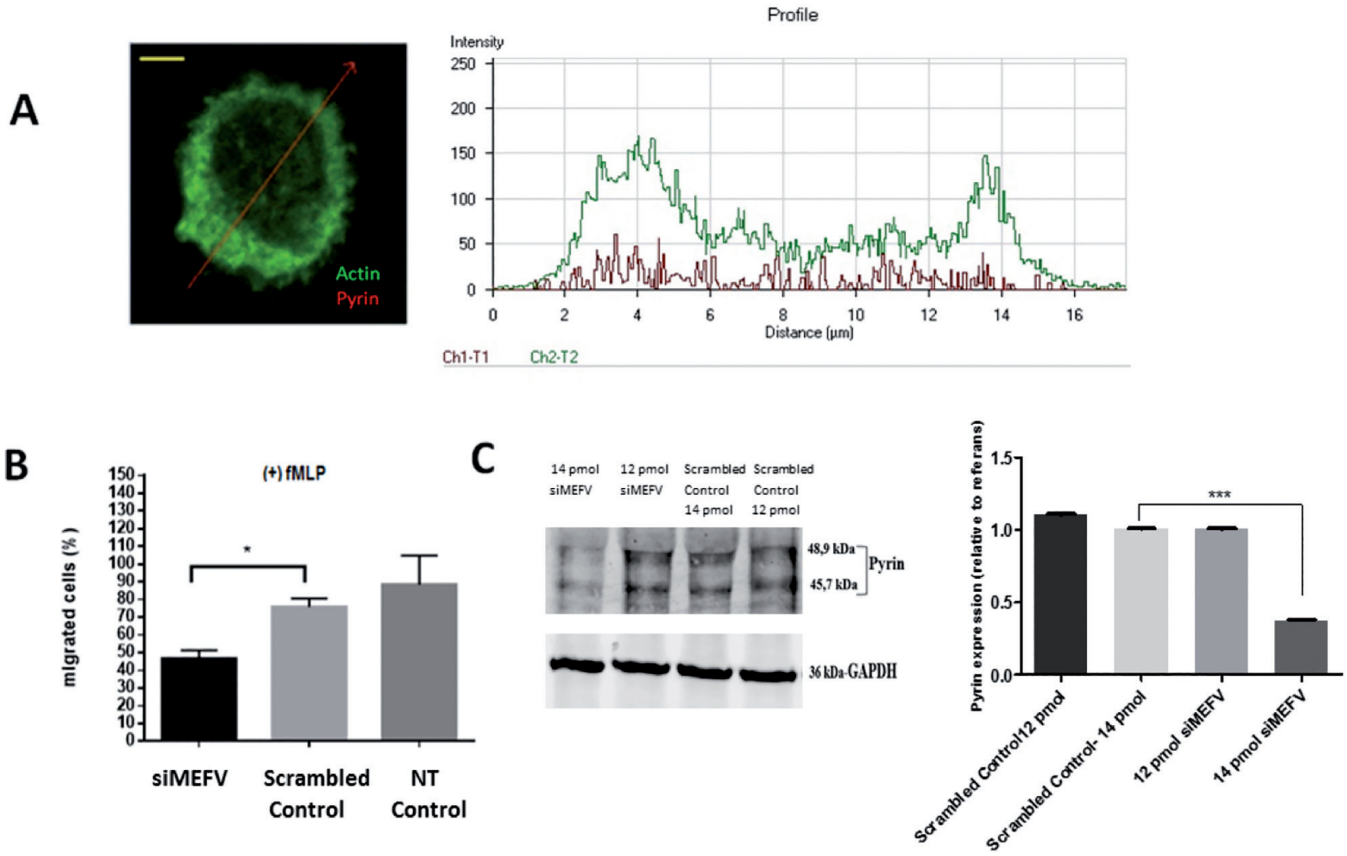

Fig. 2. Pyrin is knocked down in neutrophils-like cells. (**A**) Double staining of actin (green) and pyrin (red) in siMEFV-transfected differentiated HL-60 cells. Cells showed less polymerisation after fMLP stimulation. Scale bar = 25 µm. Profile curve shown in A is a tracing of the signal intensity (DF0) from the cell in the upper panel; the vector of the tracing is shown by the red arrow. (**B**) Percentage of migrating cells in differentiated-stimulated HL-60 after transfection with siMEFV and negative control. Cell migration was decreased significantly in pyrin-silenced HL-60 cells. Error bars indicate standard deviations. Statistically significant differences are indicated as *p<0.05. (**C**) Western Blot analysis for pyrin. The pyrin protein level is decreased compared with that in the negative control and control cells. Two bands indicated two isoforms of the MEFV gene that were translated in HL-60 cells. Quantification of Western Blot analysis for MEFV knock-down efficiency. NT: Non-transfected control. Experiments were performed biologically and technically in triplicate.

findings, chemotaxis experiments demonstrated that cell migration to the lower compartment with fMLP was decreased significantly in pyrin-silenced HL-60 cells (p<0.05) (Fig. 2B). Thus, our findings in siRNA experiments suggested that pyrin's recruitment to sites of active actin polymerisation promotes the initial process of inflammatory cell migration.

Cellular polarisation ratios and cell chemotaxis increase in FMF patients

Inflammatory migration was investigated in primary neutrophils isolated from the blood samples of 11 healthy individuals and 12 FMF patients who were homozygous for the M694V mutation. Among these patients, 4 newly diagnosed patients presented to the clinics with an attack, so their samples were studied during the attack period. The erythrocyte sedimentation rate (ESR) and level of C reactive protein (CRP) of healthy individuals and FMF patients are given in Table I. Healthy individuals whose ESR and CRP levels within normal limits were included in the study cohort. Both non-fMLP- and fMLP-mediated conditions were used to compare healthy individuals and FMF patients. The polarisation ratios in individuals were detected by phalloidin staining. The polarisation ratio of neutrophils for the fMLP-mediated condition was greater than 52% in FMF patients and 40% in healthy controls (p=0.0016) (Fig. 3 A-D). We also observed an increase in polarisation in FMF patients (38%) compared with healthy individuals (31.5%) for non-fMLP conditions, but it was not significant (p=0.1590) (Supplementary Fig. 3). The polarisation ratios were not different in patients compared with those during the attack period (data not shown).

The neutrophil migration ratio of FMF patients and healthy individuals were quantified by filter assays. Both non-fMLP- and fMLP-mediated chemotaxis in patient cells were increased significantly compared with those in healthy individuals (p<0.05) (Fig. 4A-F). We also compared different mutations with healthy controls (Fig. 5).

In addition, in the non-fMLP- and fMLP-mediated conditions, pyrin-actin co-localisation was shown in the neutrophils of FMF patients and healthy control subjects. M694V mutation did not change pyrin-actin co-localisation (Fig. 6A-F).

These results suggest that pyrin plays a crucial role as a pro-inflammatory regulator in inflammation and increases the neutrophil migration rate in FMF patients.

Table I. Erythrocyte sedimentation rate (ESR), level of C-reactive protein (CRP) and attack frequency/disease state of FMF patients with M694V/M694V mutation and healthy individuals.

Individuals involved in the study	Age	ESR (0 - 20 mm/h)	CRP (0 - 0.8 mg/dL)	Mutation	Attack frequency[a]/disease state
Patient 1	11	25	2.2	M694V/M694V	2 attacks/Attack
Patient 2	7	12	0.927	M694V/M694V	1 attack/Attack-free period
Patient 3	12	34	1.04	M694V/M694V	1 attack/Attack-free period
Patient 4	14	9	5.47	M694V/M694V	6 attacks/Attack-
Patient 5	16	5	0.256	M694V/M694V	2 attacks/Attack-free period
Patient 6	5	31	8.94	M694V/M694V	6 attacks/Attack-
Patient 7	5	23	0.605	M694V/M694V	0 attack/Attack-free period
Patient 8	15	2	0.531	M694V/M694V	4 attacks/Attack-free period
Patient 9	13	18	1.82	M694V/M694V	8 attacks/Attack-free period
Patient 10	6	6	0.191	M694V/M694V	4 attacks/Attack-free period
Patient 11	11	10	4.11	M694V/M694V	1 attack/Attack
Patient 12	14	7	0.147	M694V/M694V	0 attack/Attack-free period
Control 1	10	2	0.277	-	Healthy
Control 2	6	6	0.128	-	Healthy
Control 3	16	5	0.64	-	Healthy
Control 4	13	5	0.153	-	Healthy
Control 5	17	2	0.342	-	Healthy
Control 6	6	6	0.223	-	Healthy
Control 7	6	2	0.285	-	Healthy
Control 8	12	2	0.132	-	Healthy
Control 9	8	10	0.372	-	Healthy
Control 10	13	7	0.191	-	Healthy
Control 11	13	8	0.154	-	Healthy

[a]Number of attacks during the previous year.

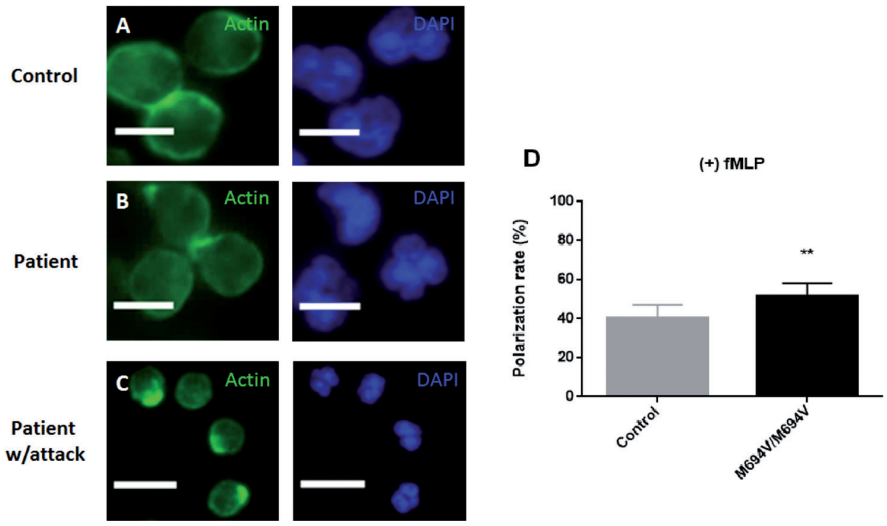

Fig. 3. Neutrophil polarisation in FMF patients and healthy individuals. (**A-C**) Double staining of actin (green) and DAPI (blue) in stimulated primary human neutrophils from healthy controls, patients and patients during attack. The scale bar for figures A and B is 5 μm and that for C is 8 μm. (D) The polarisation ratio was increased in patients (p=0.0016). For the analysis of polarisation ratio, cells were actually count at 20X and polarised/non-polarised cell number was given as percentage. Error bars indicate standard deviations. Statistically significant differences are indicated as **p<0.01. Experiments were performed technically in duplicate.

Mutations related to a severe phenotype lead to a higher increase in cell migration in FMF patients

Migration was investigated in primary neutrophils of 9 healthy individuals and 15 FMF patients with different mutations (6 M694V/M680I, 3 M694V/V726A, 3 M694V/E148Q, 3 E148Q/E148Q). The erythrocyte sedimentation rate (ESR) and level of C reactive protein (CRP) of these healthy individuals and FMF patients are given in Supplementary Table I. The neutrophil migration rate of FMF patients with different mutations and healthy individuals were analysed by filter assays. Both non-fMLP- and fMLP-mediated conditions were used to compare healthy individuals and FMF patients. The increase in the cell migration ratio was higher in patients who were homozygous for the M694V mutation and those who were compound heterozygous and had M694V in one allele (M694V/M680I*, M694V/V726A, M694V/E148Q) compared with patients homozygous for the E148Q mutation (*p<0.05) (Supplementary Fig. 4A-C). When we compared compound heterozygotes, the cell migration rate was found to be significantly higher in patients with M694V/M680I** and M694V/V726A mutations than in those with the M694V/E148Q mutation (**p<0.05) (Supplementary Fig. 5A-B). These data showed that there is an increased trend in the cell migration ratio in parallel with the severity of mutations.

Discussion

The data presented here provide new insight into the cellular function of pyrin, the protein mutated in FMF, and demonstrated an important link between pyrin and polymerising actin in the process of inflammatory cell mi-

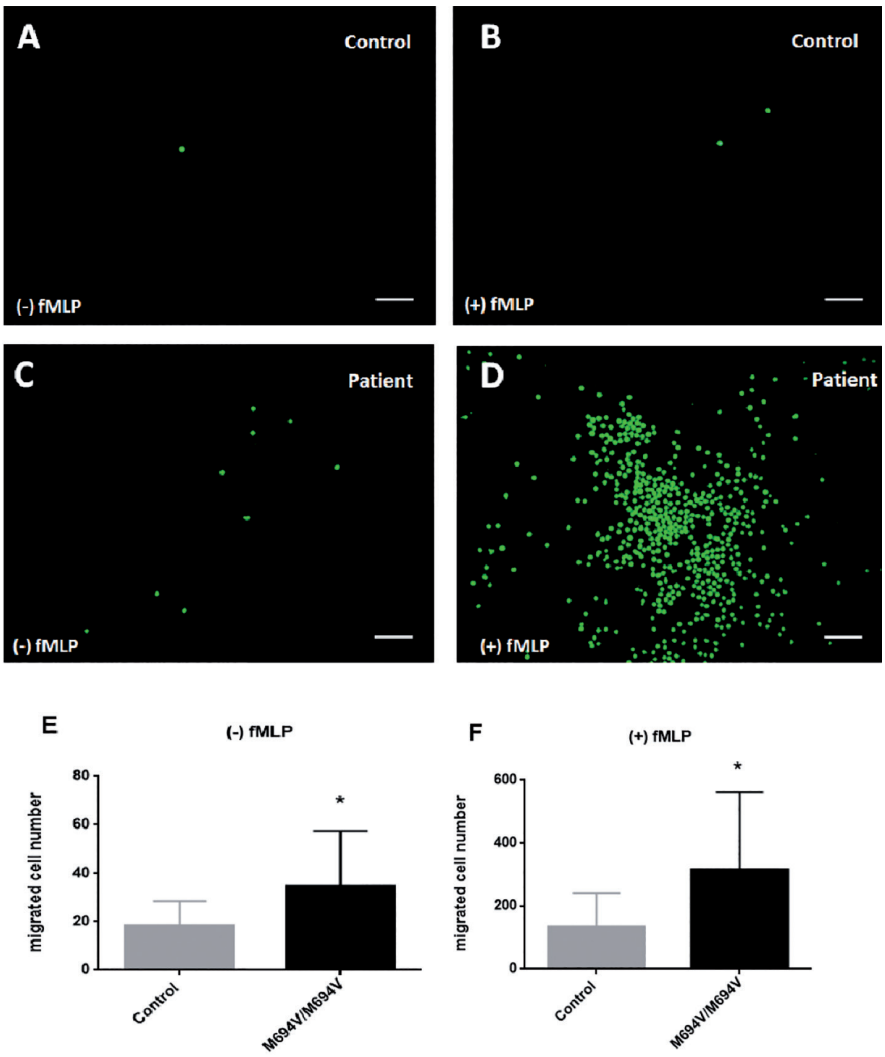

Fig. 4. Neutrophil migration ratio in FMF patients and healthy individuals. (**A** and **C**) Calcein-AM (green) staining in unstimulated neutrophils from healthy control subjects and patients who migrated to the bottom layer of the transwell chamber. (**B** and **D**) Calcein-AM (green) staining in stimulated neutrophils from healthy control subjects and patients who migrated to the bottom layer of the transwell chamber. Scale bar for figures = 20 μm. (**E**) Chemotaxis in patient cells was increased significantly compared with that in health control subjects in unstimulated cells and (**F**) stimulated cells. Error bars indicate standard deviations. Statistically significant differences are indicated as $*p<0.05$. Experiments were performed technically in duplicate.

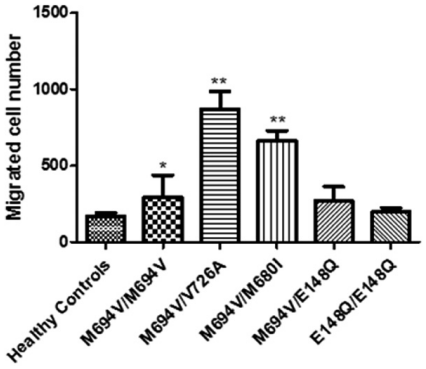

Fig. 5. Comparison of the stimulated cell migration ratio in FMF patients with the healthy controls. Error bars indicate standard deviations. Statistically significant differences are indicated as $*p<0.05$ and $**p<0.01$. Experiments were performed technically in duplicate.

gration. Previous work concerning the pyrin-actin relationship revealed an association of pyrin with active actin polymerisation sites in human monocytes and *Listeria* rocket tails (19). In agreement with these data, we have shown that pyrin co-localises with polymerising actin at the leading edge in migrating human neutrophils, which are the main cell type expressing pyrin.

Actin is a major cytoskeletal protein and, with its associated proteins, plays a central role in the regulation of cell polarisation and movement. It was proposed that, for these processes, waves of actin filaments propagate within various types of cells, including neutrophils (26). Actin-related molecules, such as VASP and Arp2, and other pyrin-related proteins have been shown to be enriched in actin waves in neutrophils and macrophages (26). Another study has demonstrated that the binding of pyrin to VASP and Arp3 (19) supports the idea of pyrin's possible role in these regions of actin waves because Arp3 works together with Arp2 to nucleate actin filaments at the minus end, allowing growth at the plus end. Furthermore, the Arp2/3 complex can nucleate a branch from an existing actin filament, allowing the formation of actin filaments (27). Our cell migration modeling on knocked-down pyrin cells resulted in a dramatic reduction in inflammatory cell migration in neutrophils-like cells. These data highlighting the requirement of pyrin to be recruited to sites of active actin polymerisation for the initial process of inflammatory cell migration, together with the interaction data, support the idea that pyrin can be considered as another cell-specific, actin-related molecule, mainly functioning in neutrophil migration.

In a previous study, it was demonstrated that, in response to inactivating modifications of Rho GTPases by various bacterial toxins or effectors, pyrin forms a caspase-1-activating inflammasome, which correlated with the dephosphorylation of mouse pyrin (28). In another study, RhoA activity was shown to suppress pyrin inflammasome activation, which was important in terms of revealing the downstream effector serine-threonine kinases PKN1 and PKN2 that bind and phosphorylate pyrin (12). These previous demonstrations of pyrin's connection with Rho GTPases are important because this protein family is responsible for the organisation and regulation of the actin cytoskeleton, and, consequently, many actin-related cellular processes like cell migration (29, 30). Functional studies have shown that the depletion of RhoD, a Rho GTPase member, leads to increased actin filament-containing structures and defects in important cellular functions such as

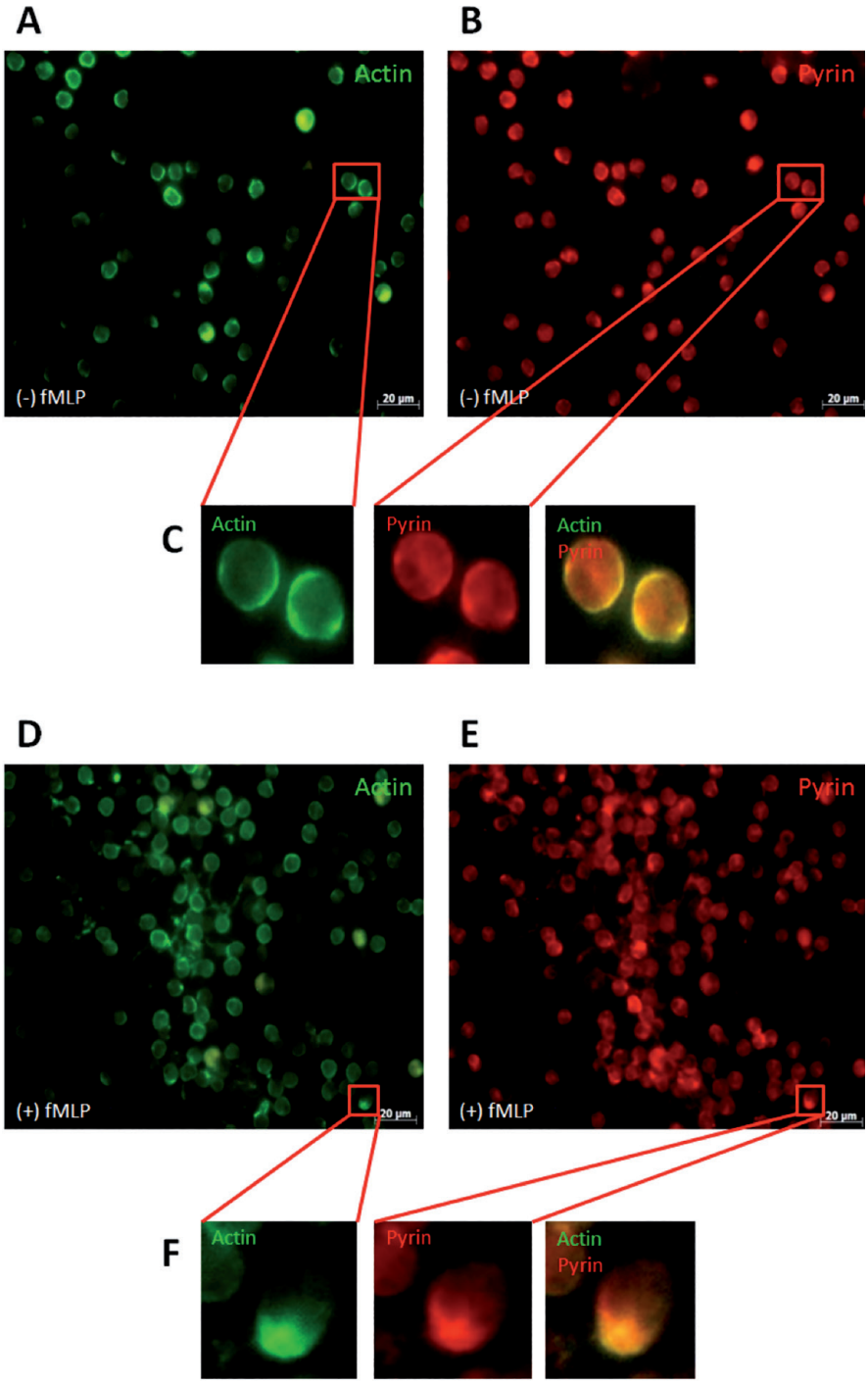

Fig. 6. Pyrin-actin co-localisation in human neutrophils isolated from M694V homozygote patients. (**A-B**) Double staining of actin (green) and pyrin (red) in unstimulated primary human neutrophils isolated from M694V homozygote patients. (**C**) Selected cell from A and B for zoom-in view. (**D-E**) Double staining of actin (green) and pyrin (red) in stimulated primary human neutrophils isolated from M694V homozygote patients. (**F**) Selected cell from D and E for zoom-in view. M694V mutation did not affect pyrin-actin co-localisation. Scale bar for figures = 20 µm. Experiments were performed technically in duplicate.

cell migration and proliferation (29). Thus, our data concerning pyrin's interaction with actin and increase in cell migration in FMF patients, in accordance with previous data regarding pyrin's connection with RhoGTPases, strengthens the possible role of pyrin in the actin machinery.

Although the effects of pyrin mutations remain controversial regarding whether FMF conditions are due to the loss of function in the inhibition of inflammation or gain-of-function in pro-inflammation, publications in recent years have provided evidence for an increase in inflammasome activation in pyrin mutants to support the idea of a gain-of-function (31, 32). In agreement with these publications, our observation of a dramatic increase in cellular polarisation and migration in the cells of FMF patients under *in vitro* conditions not only supported the gain-of-function hypothesis but also showed that the differential capacity of cell migration is affected by intrinsic factors. Our findings where the cell migration rate is increased dramatically in severe types of mutations, such as M694V and M680I, in patient's neutrophils are also consistent with the genotype-phenotype correlations explained so far (33, 34). Recently, miRNAs, miR-20a-5p, miR- 197-3p, were found to be differentially regulated in homozygote FMF patients. Bioinformatics analysis revealed that these miRNAs target mRNAs clustered in inflammatory pathways such as cell migration which may be another supportive information for the difference in cell migration seen in patients (35).

In conclusion, we have shown that pyrin has a strong potential to modulate inflammatory cell migration through its interaction with polymerised actin in neutrophils. However, the exact signaling mechanism underlying the activation of the pyrin inflammasome during cell migration remains unclear and deserves further investigation.

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