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The Effect of Colchicine on Pyrin and Pyrin Interacting Proteins

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ABSTRACT

MEFV which encodes pyrin, cause familial Mediterranean fever (FMF), the most common auto-inflammatory disease. Pyrin is believed to be a regulator of inflammation, though the nature of this regulatory activity remains to be identified. Prophylactic treatment with colchicine, a microtubule toxin, has had a remarkable effect on disease progression and outcome. It has been thought that, inhibition of microtubule polymerization is the main mechanism of action of colchicine. But, the exact cellular mechanism explaining the efficacy of colchicine in suppressing FMF attacks is still unclear. Given the ability of colchicine treatment to be considered as a differential diagnosis criteria of FMF, we hypothesized that colchicine may have a specific effect on pyrin and pyrin interacting proteins. This study showed that colchicine prevents reticulated fibrils formed by PSTPIP1 filaments and reduces ASC speck rates in transfected cells. We further noted that, colchicine down-regulates *MEFV* expression in THP-1 cells. We also observed that colchicine causes re-organization of actin cytoskeleton in THP-1 cells. Pyrin is an actin-binding protein that specifically localizes with polymerizing actin filaments. Thus, *MEFV* expression might be affected by re-organization of actin cytoskeleton. The data presented here reveal an important connection between colchicine and pyrin which might explain the remarkable efficacy of colchicine in preventing FMF attacks. J. Cell. Biochem. 113: 3536–3546, 2012.

KEY WORDS: COLCHICINE; PYRIN; ASC; PSTPIP1; INFLAMMATION; MEFV

Familial Mediterranean fever (FMF) (MIM 249100) is the most common of the auto-inflammatory disorders. It is characterized by recurrent attacks of fever and localized inflammation, usually involving the peritoneum, pleura, joints, and skin [Livneh et al., 1997]. A rapid acute phase response and a massive influx of polymorphonuclear leukocytes into the affected tissues cause the inflammation in FMF patients. Tissue deposition of serum amyloid A can lead to kidney failure and death [Livneh et al., 1999]. FMF is caused by mutations in the *MEFV* (MEditerranean FeVer) (OMIM: 608107) [The International FMF Consortium, 1997]. So far more than 74 FMF-associated mutations have been identified in the *MEFV* (http://fmf.igh.cnrs.fr/infevers/). Pyrin (NP_000234.1), the product of *MEFV*, is expressed predominantly in neutrophils, monocytes, and dendritic cells, but not in lymphocytes [Centola et al., 2000; Diaz et al., 2004].

The pyrin protein appears to be a regulator of inflammation, but its exact role on inflammatory pathways is still controversial. Pyrin is composed of five domains, each of which has a distinct role in interactions with several proteins that are related to inflammation through regulation of cell death, regulation of cytokine secretion, and cytoskeletal signaling [Chae et al., 2009].

Several pyrin-interacting proteins have been identified, including ASC (NP_037390.2) [Richards et al., 2001], PSTPIP1 (NP_003969) [Shoham et al., 2003], and Siva (NP_006418.2) [Balci-Peynircioglu et al., 2008a].

PSTPIP1 (Proline Serine Threonine Phosphatase-Interacting Protein 1) is a cytosolic adaptor protein that functions to link PEST phosphatases to their substrates [Cong et al., 2000]. PSTPIP1 is distributed in a filamentous network throughout the cytosol in both natively expressing and transfected cells [Waite et al., 2009a]. Mutations in the *PSTPIP1* result in PAPA Syndrome, (Pyogenic sterile Arthritis, Pyoderma gangrenosum, and Acne) (MIM 604416) [Wise et al., 2002]. PAPA-associated mutants of *PSTPIP1* are able to form filaments normally in human monocytes, neutrophils, and also

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transfected cells. Our previous work demonstrated that pyrin reorganizes PSTPIP1 fibrils so that they become branched and reticulated in the presence of pyrin in transfected cells [Waite et al., 2009a]. This indicates that pyrin plays a role in remodeling the PSTPIP1 fibrils when it binds to them. The biological impact of reticulated fibrils is as yet unknown, but it is thought that this well-organized structure may play an important role in immune cells.

Pyrin also interacts with ASC (Apoptosis-associated Speck-like protein containing a Caspase recruitment domain) via a PYD–PYD interaction. ASC is an adaptor protein that is an important component of both the apoptosome and the inflammasome [Bergsbaken and Cookson, 2009]. It forms large perinuclear structures called "specks" in vitro [Masumoto et al., 1999]. We previously demonstrated that speck formation is accelerated in the presence of pyrin, and we also found that nocodazole, which inhibits microtubule formation and decreases speck formation [Balci-Peynircioglu et al., 2008b].

The most effective treatment for FMF is the daily use of colchicine [Zemer et al., 1986], which reduces both the frequency and severity of FMF attacks, and reduces the incidence of multi-organ amyloidosis. Colchicine forms a tubulin–colchicine complex causing microtubule de-polymerization. This has been thought to be the main effect of colchicine in treating FMF, however how colchicine suppresses FMF attacks is still unclear [Niel and Scherrmann, 2006]. We hypothesized that colchicine may have some specific effects on pyrin and/or pyrin-interacting proteins. Indeed, the effect of colchicine on these proteins and on gene expression levels of these proteins was previously unknown.

In this study, we determined the effect of colchicine on distribution and expression of pyrin and its interacting proteins. We firstly investigated the effect of colchicine on a cellular level by examining possible differences in reticulated fibril structures in pyrin-PSTPIP1 co-transfected cells, speck formation in pyrin-ASC co-transfected cells. We then evaluated the results of transcription levels in the presence or absence of LPS. The results of this study provide a detailed outline of the effect of colchicine on the gene expression levels and cellular distribution of these proteins.

METHODS

CELL CULTURE, TRANSFECTION, AND COLCHICINE TREATMENT

COS-7 and HeLa cells were grown in DMEM (Gibco by Invitrogen, Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum) (vol/vol), and 1% L-glutamine (vol/vol), and then transfected using FUGENE-HD (Roche Applied Science, Indianapolis, IN).

For COS-7 and HeLa cells, 6 h after being transfected with the vectors, cells were treated with colchicine at microtubule non-disrupting concentrations which is determined according to experiments with different concentrations of colchicine (Biological Industries, Kibbutz, Israel). In these experiments, COS-7 and HeLa cells were treated with varying concentrations; 1–100 ng/ml and 1–50 ng/ml of colchicine containing mediums; respectively. Cells were fixed and stained 18 h after colchicine treatment. For analysis, 500 cells were counted in triplicates. THP-1 cells were grown in RPMI (Gibco by Invitrogen) supplemented with 10% FBS (vol/vol), and 1% glutamine (vol/vol). Cells were differentiated with PMA as previously described in [Daigneault et al., 2010]. In order to determine microtubule non-disrupting concentrations for THP-1 cells, they were treated 100–1,000 ng/ml of colchicine-containing medium for 24 h after starting with the 7th day of differentiation. THP-1 cells were also treated with 10 ng/ml LPS (Sigma–Aldrich, Steinheim, Germany) where indicated. All experiments were performed in triplicate. All cell lines were obtained from ATCC (American Type Culture Collection).

PLASMIDS AND ANTIBODIES

All FLAG-tagged, myc-tagged, and GFP tagged constructs were provided by the laboratory of Dr. Deborah Gumucio (University of Michigan); these constructs were generated using pCMV-Tag2B, pCMV-Tag3A (from Staratagene, La Jolla, CA) and pEGFP-C2 (from Clontech, Mountain View, CA) vectors, respectively. Anti-myc (rabbit polyclonal), anti-FLAG (Cy3 conjugate mouse antibody), and anti-tubulin (mouse monoclonal) antibodies were obtained from Sigma (St. Louis, MO). Actin filaments were visualized using AlexaFluor488 Phalloidin from Molecular Probes by Invitrogen (Eugene, OR). Fluorescent-labeled secondary antibodies (Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit) were also obtained from Molecular Probes by Invitrogen. Anti-Pyrin (Abnova Corporation, CA) polyclonal antibody and anti-GAPDH (Applied Biosystems/Ambion, Inc., Austin, TX) monoclonal antibody were used for Western Blot analysis. Horseradish peroxidase (HRP)-conjugated immunoglobulin-G was purchased from Invitrogen-Molecular Probes, Carlsbad, CA.

IMAGING ANALYSIS

COS-7 cells were co-transfected with PSTPIP1-GFP and myc-pyrin. A PSTPIP1-GFP positive region was found and followed for 24 h. GFP and PSTPIP1-GFP transfected COS-7 cells were used as control in the analysis. In the time-lapse imaging, cells were maintained at 37°C under the microscope and imaged every 15 min during the experiment.

THP-1 cells were followed for 24 h after treatment with 100 ng/ml colchicine and then another 24 h in fresh medium after removal of colchicine containing medium in order to determine the effect of colchicine on filopodia length.

Leica Application Suite was used to analyze the time-lapse image sequences. For imaging and measurement Leica QWin image analysis and processing software for quantitative microscopy was used.

IMMUNOFLUORESCENCE STAINING

Cells were fixed for 30 min in 4% paraformaldehyde solution in PBS at room temperature. After permeabilization in 0.2% Triton-X in PBS for 10 min, cells were exposed to blocking solution (10% goat serum, 2% BSA, and 0.1% Tween-20 in PBS) for 1 h. After antibody staining, 10 mM DAPI was applied to the cells for 1 min for nuclear visualization. Coverslips were mounted with ProLong Gold Anti-Fade Reagent (Molecular Probes by Invitrogen). Slides were visualized using a Leica IM 50 fluorescence microscope.

RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was extracted by Qiagen (Valencia, CA) RNeasy Mini kit according to manufacturer's instructions. Once total RNA was obtained, its concentration and OD ratio was measured by NanoDrop ND 1000 (Thermo Scientific, Waltham, MA). cDNA was generated by reverse transcription of 500 ng total RNA using Qiagen Quanti Tect Reverse Transcription kit according to manufacturer's recommendations.

QUANTITATIVE REAL TIME PCR

Real-time PCR was performed using SYBR Green JumpStart TaqReadyMix kit (Sigma) on Corbett Rotor Gene 6000 Light Cycler. The relative amount of mRNA, normalized to an internal control *ACTB* (NM_001101.3) (human beta-actin) and relative to a calibrator (normal), was calculated by $2^{-\Delta\Delta CT}$. The sequences, amplicon lengths, and Tm degrees of gene specific primer pairs for *ACTB*, *MEFV*, *ASC* (NM_013258.4), *PSTPIP1* (NM_003978) and *Siva* (NM_006427.3) are shown in Table I. All reactions were performed in 95–100% efficiency.

WESTERN BLOT

THP-1 cells were lysed in Triton X-100 Lysis Buffer (10 mM Tris-Base at pH 7.4, 300 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 in the presence of protease-inhibitor cocktail (Complete Mini, EDTA-free protease inhibitor cocktail tablets, Roche). Protein concentrations were determined using BCA assay kit (Thermo Fisher Scientific, Inc., Waltham, MA) according to manufacturer's instructions. Equal amounts of total protein (40 μg/ml) were denatured in SDS sample buffer, subjected to 12% SDS-PAGE and transferred to nitrocellulose (NC) membrane (Thermo Scientific) for detection with appropriate antibodies.

STATISTICAL METHOD

Statistical analysis was performed by using GraphPad Prism ver 5.01 (GraphPad Software, Inc., San Diego, CA). *P* values were calculated by using one-way ANOVA test and Tukey's test was used as the post test where *P*-values of <0.05 were considered statistically significant.

TABLE I. Primer Sequences Used in Real-Time PCR Experiment

Gene	Primer sequences	Amplicon length (bp)
ACTB	5′-CGCAAAGACCTGTACGCCAAC-3′ 5′-GAGCCGCCGATCCACACG-3′	164
MEFV (Ex2–3)	5′-TGGACTCGGCAACAGAACC-3′ 5′-CGTCAACTGGGTCTCCTTCC-3′	162
MEFV (Ex4–5)	5′-GGGAGGAGAAGGCAGTGAG-3′ 5′-AGCAGGGCGATGTCCTGGG-3′	192
ASC	5′-CTTCTACCTGGAGACCTACG-3′ 5’-CGGTGCTGGTCTATAAAGTG-3’	183–240[a]
PSTPIP1	5′-GCAGCATAGACGCCGACATC-3′ 5′-CTTCCGTGCAGCAGTCCAG-3′	178
Siva	5′-TGCGTGCGAGCCGTGGATG-3′ 5′-GCTTGAGCCAGCCTCAGGTCTC-3′	193

Ex, exon; bp, base pair.
[a]Amplification products depend on alternative splicing.

RESULTS

DETERMINATION OF SUITABLE COLCHICINE CONCENTRATION

In order to determine the specific effects of colchicine besides microtubule depolymerization, we treated cells with levels of colchicine below microtubule-disrupting concentrations. In order to determine the appropriate concentration, cells were treated with different concentrations of colchicine and microtubule architecture was visualized by immunofluorescence with a tubulin-specific antibody. The intact microtubular architecture, characterized by filamentous microtubules spreading from a perinuclear tubulin-dense microtubule organizing center, was observed in colchicine-free medium. Microtubule disruption was observed at colchicine concentrations higher than 7.5, 10, and 100 ng/ml colchicine concentrations for COS-7, HeLa, and THP-1 cells, respectively (Fig. 1A–C). At lower colchicine concentrations microtubular architecture was similar to those of cells grown in colchicine-free medium. We also performed actin filament staining by chemi-fluorescence and observed that the actin cytoskeleton was intact at colchicine concentrations mentioned above. However, an increase in number of stress fibers were observed in THP-1 cells when treated with 250 ng/ml colchicine-containing medium (Fig. 2A–D). We further noted that filopodia were decreased in both the average length and number in differentiated THP-1 cells after colchicine treatment (Fig. 2E–H). We also performed time lapse live cell imaging in 100 ng/ml colchicine containing medium in THP-1 cells, and observed a decrease in filopodia upon colchicine treatment (Fig. 3A). In the 24th hour of colchicine treatment, cells were washed three times with fresh medium and treated with culture medium without colchicine (Fig. 3B). Following colchicine removal filopodia re-formed, indicating that filopodia formation was reversibly altered by colchicine. These observations indicate that colchicine, at low concentrations, causes actin re-organization while leaving microtubules intact.

THE FORMATION OF RETICULATED FIBRILS

In order to analyze the formation of reticulated fibrils in COS-7 cells after co-transfection with myc-pyrin and PSTPIP1-GFP, we selected a field containing GFP-positive cells and followed them by time lapse live cell-imaging. At the 18th hour after transfection, GFP expression was first observed as un-organized aggregates; however, these aggregates became branched and organized at 20th hour, as shown in Figure 4A3. However reticulated fibril formation was not observed when cells were transfected with either GFP only or PSTPIP1-GFP (Fig. 4A1–A2). This led us to conclude that reticulated fibrils form in a short period after the accumulation of transfected proteins in COS-7 cells.

COLCHICINE DECREASES THE NUMBER OF RETICULATED FIBRIL FORMING CELLS

It is known that PSTPIP1 forms filamentous structures in transfected and native human cells. First, we investigated the effect of colchicine on PSTPIP1 filaments in flag-PSTPIP1 transfected COS-7 cells. However, we did not observe any differences between control and colchicine treated cells (data not shown).

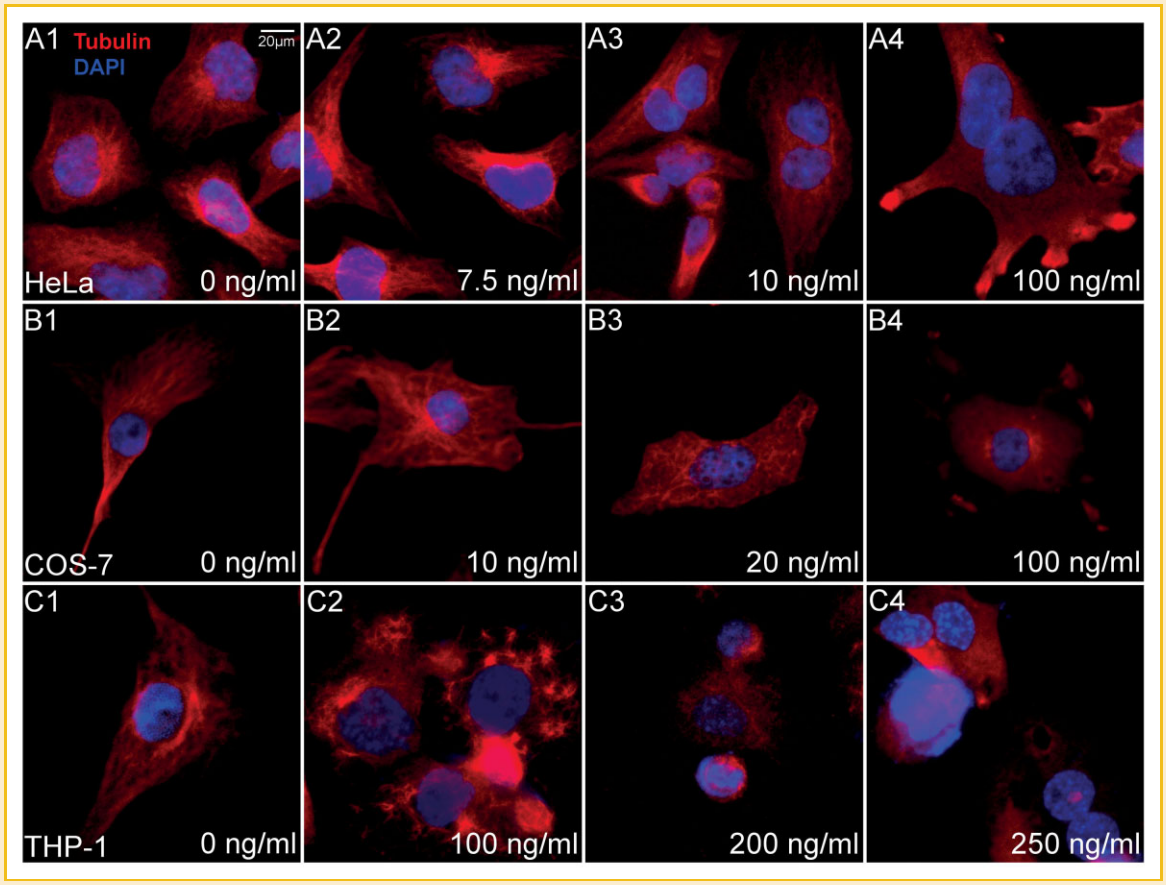

Fig. 1. Effects of colchicine on microtubule structure at various concentrations. Tubulin (red) and nucleus (blue) staining of HeLa cells (A1–A4) incubated for 24 h in mediums containing 0, 7.5, 10, and 100 ng/ml colchicine, COS-7 cells (B1–B4) incubated for 24 h in medium containing 0, 10, 20, and 100 ng/ml colchicine, and differentiated THP-1 cells (C1–C4) incubated for 24 h in medium containing 0, 100, 200, and 250 ng/ml colchicine. Microtubule disruption is observed at 10, 20, and 200 ng/ml colchicine concentrations for HeLa, COS-7, and THP-1 cells, respectively. Below these concentrations, microtubule architectures were similar with control cells incubated in medium containing no colchicine, with a proper microtubule organization center proximal to nucleus and individually distinctive microtubule filaments. Scale bar for figures A–C shown in A1 = 20 μm.

To determine the effect of colchicine on reticulated fibrils, COS-7 cells were co-transfected with myc-Pyrin and flag-PSTPIP1. In this assay, co-transfected cells were classified as either having non-reticulated or reticulated PSTPIP1 architecture (Fig. 4B). As shown in Figure 4C, reticulated fibrils formed in 60% of co-transfected cells. In colchicine treated group, however, the proportion of cells that have reticulated fibrils was reduced by 10% ($P < 0.001$).

We also tested the effect of FMF-causing mutations on pyrin in the presence or absence of colchicine. The results were similar for COS-7 cells co-transfected with flag-PSTPIP1 (wild type) and myc-pyrin-M694V when compared with cells co-transfected with flag-PSTPIP1 and wild-type pyrin; colchicine treatment also caused a decrease in reticulated fibril ratio when mutant pyrin was transfected ($P < 0.001$).

We then set out an experiment to determine the effect of a PAPA-causing mutation on fibril formation. We co-transfected COS-7 cells with flag-tagged PSTPIP1 containing the A230T mutation, a known PAPA mutation, and myc-tagged pyrin. We found that a significantly higher proportion of cells (75%) contained reticulated fibrils with mutant PSTPIP1 ($P < 0.001$; Fig. 4C). However, when these cells were treated with colchicine, no significant reduction in fibril formation was observed.

Therefore, our findings suggest that colchicine may prevent reticularization of PSTPIP1 because of the connection between PSTPIP1 filaments and the cellular cytoskeleton which results in a decrease in the number of reticulated fibril forming cells.

ASC SPECK FORMATION IS FACILITATED BY PYRIN AND DISRUPTED BY COLCHICINE

Next, we planned to determine the effects of different forms of pyrin on ASC speck formation, and how this rate is affected by colchicine. Co-transfection assays were carried out in HeLa cells instead of COS7 cells as ASC expressing COS7 cells quickly disappeared from culture medium due to probable apoptosis trigerred by ASC oligomerization. For each assay, either wild type pyrin or one of three FMF-causing pyrin mutants (M694V, M680I, or V726A, all myc-tagged) was co-transfected with ASC-YFP. The assay was terminated in the 19th hour after transfection due to increased cell death as a result of the apoptotic effects of ASC. All the cells included in the analysis had a normal nuclear morphology.

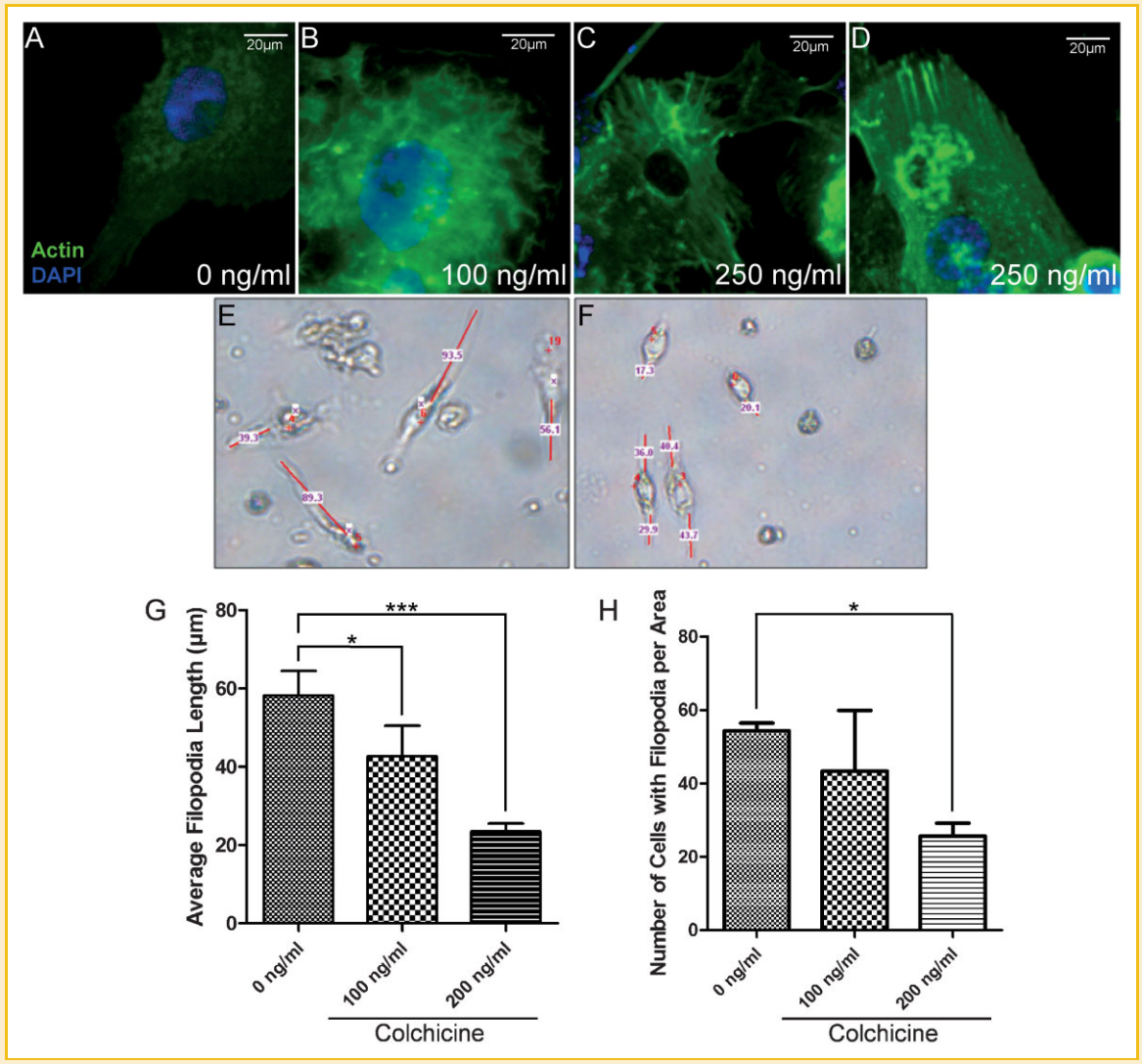

Fig. 2. Actin cytoskeleton and filopodia properties of differentiated THP-1 cells treated with colchicine. Following the treatment of differentiated THP-1 cells with various concentrations of colchicine containing medium, cells were stained for nucleus (blue), and actin (green) with Phalloidin-488 (A–D). Stress fibers were observed with 250 ng/ml colchicine administration (C–D) where this was not a common occurance in lower colchicine concentration (100 ng/ml) (B), and no colchicine (A). Scale bars in A–D shows 20 μm. E–F: Show the measurement of filopodia length. Statistical analysis indicates that 100 and 200 ng/ml colchicine caused a contraction of filopodia in differentiated THP-1 cells (G) and cells with filopodia are significantly lower in number when treated with 200 ng/ml colchicine (H). Error bars indicate standard deviations. The statistically significant differences are indicated as $^{*}P < 0.05$, $^{***}P < 0.001$.

Co-transfected cells were classified according to their intracellular ASC expression patterns as either speck forming or having diffuse ASC localization (Fig. 5A). We determined that pyrin (both the wild type and all mutants) increased end-point speck formation rate of ASC, compared to ASC alone and the control (pcDNA3.1) group (Fig. 5B). Although pyrin-M680I and pyrin-V726A groups did not increase speck formation at a level of statistical significance ($P = 0.251$ and $P = 0.171$, respectively), pyrin-M694V caused a significant increase in the speck formation rate compared to the wild type pyrin ($P = 0.021$). When co-transfected cells were exposed to colchicine, speck formation decreased with all mutant forms of pyrin and with pcDNA 3.1 ($P < 0.0001$). However, the rate at which cells containing wild-type pyrin formed specks was not significantly affected by the presence of colchicine.

These data once more showed that ASC speck formation is facilitated by pyrin and reduced by colchicine in mutant pyrin expressing cells which is important given the known beneficial effects of colchicine in preventing FMF attacks.

COLCHICINE DECREASES PYRIN EXPRESSION IN DIFFERENTIATED THP-1 CELLS

We further analyzed whether colchicine influences the expression of pyrin and its interacting proteins in natively expressing cells. We used THP-1 cell line since COS7 and HeLa cells do not naturally express pyrin and its interacting proteins, PSTPIP1, ASC, and Siva. Different concentrations of colchicine; 5, 10, 25, 50, 75, 100, 150, 200, and 250 ng/ml were used to treat differentiated (macrophage-like) THP-1 cells. Gene expression was measured

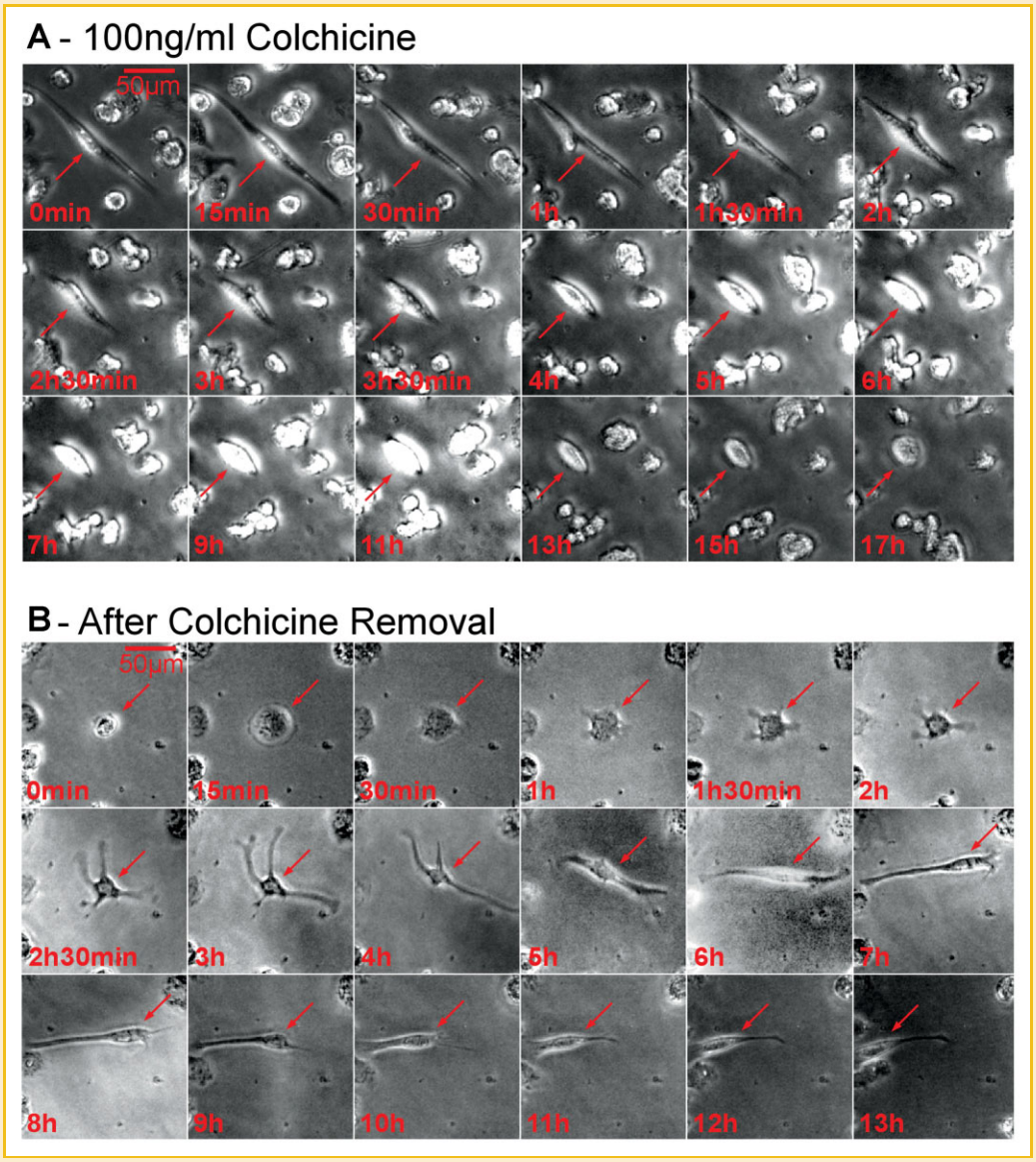

Fig. 3. Disruption and re-organization of filopodia in THP-1 cells. Phase contrast time-lapse imaging of differentiated THP-1 cells were performed and shown as pictures various time points as indicated. In medium containing 100 ng/ml colchicine (A), contraction of filopodia began immediately in the 1st hour. By the 4th hour of administration filopodia was totally contracted for this cell (indicated with arrow). In 17th hour of the experiment this cell totally lost its fusiform structure and assumed a round shape. B shows a round shaped differentiated THP-1 cell, priorly treated with 100 ng/ml colchicine, washed various times with fresh medium and now resting in medium with no colchicine. Several cell appendages begin to protrude from the main body by the end of the 1st hour. Around the 5th to 6th hour one of these appendages dominate and the cell assumes a spindle-like shape. At this time point the cell (indicated with arrow) also begins to migrate toward the left side of the area, with contraction of the filopodia that can be clearly seen from the 8th to 11th hour. This indicates a functional filopodia capable of promoting cell migration has developed for this cell after colchicine removal. Scale bars indicate 50 μm.

using quantitative RT-PCR, and protein levels were measured using Western blot.

As shown in Figure 6A, a dramatic decrease in *MEFV* was observed compared to control cells at and above 100 ng/ml colchicine concentrations. Western blot analysis showed a decrease in protein expression similar to the decrease in transcription (Fig. 6B). A previous study has shown that LPS (bacterial lipopolisaccharide) treatment induces *MEFV* expression [Centola et al., 2000]. We confirmed that this is the case in our system. The addition of 100 ng/ml colchicine did not change the level of *MEFV* up-regulation in response to LPS. However, exposure to 200 ng/ml of colchicine partially abrogated the LPS effect (Fig. 6A). We then quantified the *MEFV* expression at different treatment time points. *MEFV* were found to be down-regulated in the 6th hour of colchicine administration (Fig. 6C).

It is known that the protein product of an alternative splice variant of *MEFV* transcript lacking exon 2 locates in nucleus rather than cytoplasm [Papin et al., 2000]. In order to determine whether colchicine affects the production of this variant, we performed qRT-PCR on either control THP-1 cells, or those that had been treated

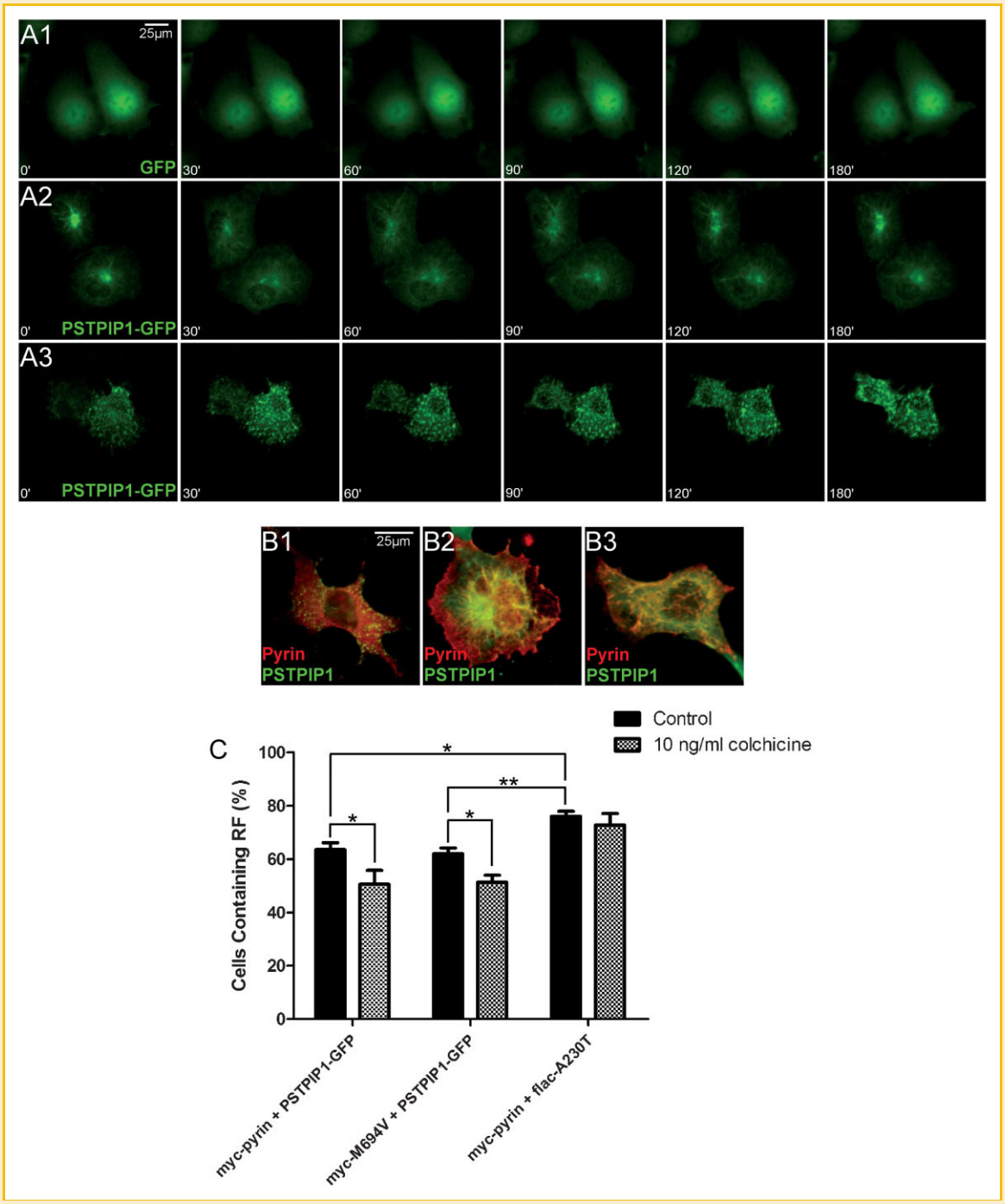

Fig. 4. PSTPIP1 reticulated fiber formation and effects of pyrin/PSTPIP1 mutants and colchicine in COS-7 cells. Time-lapse fluorescent images of COS-7 cells transfected with GFP (A1), PSTPIP1-GFP (A2), myc-pyrin, and PSTPIP1-GFP (A3) is shown in different time points as indicated. The 0' time point indicates the 18th hour of transfection. In A1 GFP is distributed in the cytoplasm as well as in the nuclei of the cells, without forming any organized structures. Unlike GFP, PSTPIP1-GFP gradually forms straight filaments in the cytoplasm originating from a single perinuclear region (A2). As a result of pyrin co-transfection, PSTPIP1-GFP gradually organizes within the cells by the 2nd hour of imaging and assumes a completely reticulated appearance by the 3rd hour (A3). Various intracellular structures in COS-7 cells co-expressing pyrin and PSTPIP1 are shown in (B); irregular PSTPIP1 aggregates (B1), PSTPIP1 fibers originating from a single perinuclear center (B2), reticulated PSTPIP1 fibrils co-localized with pyrin (B3). C: shows the percentage of reticulated PSTPIP1 fibril containing cells among transiently pyrin/PSTPIP1 co-transfected COS-7 cells. Error bars indicate standard deviations. The statistically significant differences are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars indicate 25 μm.

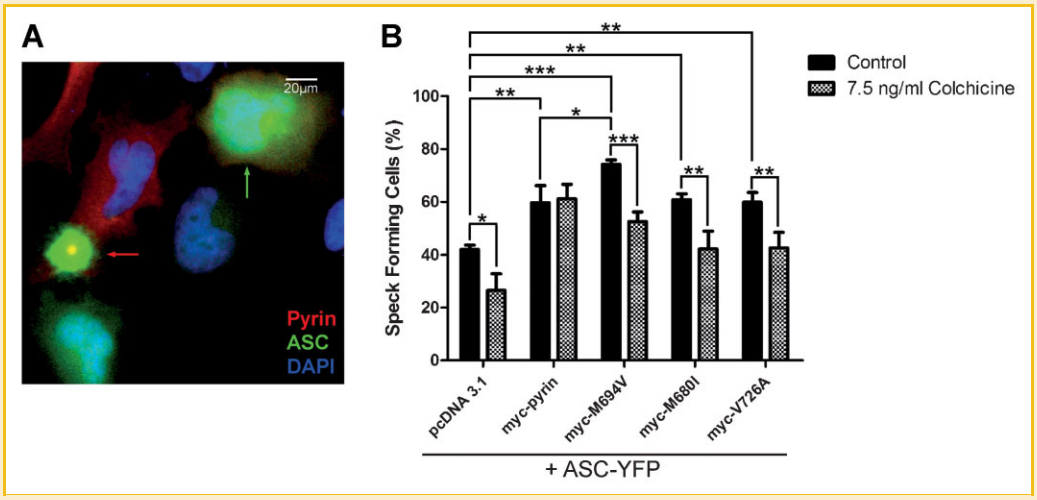

Fig. 5. Cellular localization of ASC and effects of pyrin mutants and colchicine on speck formation. In (A), two possible localization of ASC protein in myc-pyrin/ASC-YFP co-transfected HeLa cells is shown. The red arrow indicates a co-transfected HeLa cell with an ASC speck while the green arrow indicates a co-transfected cell showing a diffuse ASC distribution pattern both in the cytoplasm and nucleus. B shows the percentage of ASC speck forming cells among transiently pyrin/ASC co-transfected HeLa cells. ASC-YFP and wild-type (myc-pyrin) or mutant pyrin (myc-M694V, myc-M680I, and myc-V726A) transfected HeLa cells in the presence or absence of colchicine are indicated. Error bars indicate standard deviations. The statistically significant differences are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars indicate 20 μm.

with colchicine using a primer pair that hybridizes to exon 2 and 3 (Table I). There was no difference between those two transcripts in terms of expression. Indeed, we did not quantify the amount of exon 2 skipped variant of *MEFV* since we just needed to check if a change in pyrin expression level is coming from a common variant or not, but our immunostaining study showed that pyrin is located only in the cytoplasm in both control and colchicine treated THP-1 cells (data not shown).

In contrast to pyrin, the transcription levels of *ASC*, *PSTPIP1*, and *Siva* were not affected by colchicine administration (Fig. 6D–F). However, like *MEFV*, *ASC*, and *PSTPIP1* were significantly up-regulated by LPS stimulation (4-fold and 1.5-fold, respectively). *Siva* transcription levels were not found to be changed by LPS.

Therefore, our findings showed that colchicine have a specific effect on *MEFV* expression as we expected since colchicine treatment is a criterion for the differential diagnosis of FMF.

DISCUSSION

In this study, we characterized the effect of colchicine on Pyrin and its interacting proteins on cellular level using transfection models in COS-7 and Hela cells, and gene expression profiles in THP-1 cells. The unifying theme of all of the assays was the efficacy of colchicine for the treatment of FMF and the central question being: why is colchicine effective? The answer of this question may be summarized as follows according to our data: Colchicine may be working on several levels; (i) to reduce ASC speck formation as shown by other studies as well, using nocadozole, another microtubule depolymerazing agent, (ii) to alter intracellular distribution of PSTPIP1 which is an important effect given the fact that there is a strong connection between PSTPIP1 filaments and the cytoskeleton, (iii) to alter actin on a cellular level, and (iv) to alter pyrin expression.

In this study, we first showed that reticulated fibril ratio was decreased by colchicine in Pyrin-PSTPIP1 co-transfected COS-7 cells. Although this decrease was by %10, the biological significance has to be investigated. Secondly we found that colchicine also decreases reticulated fibril formation when cells are transfected with Pyrin-M694V and wild type PSTPIP1. On the other hand, colchicine had no effect on reticulated fibril pattern when the cells were transfected with PSTPIP1-A230T and wild type Pyrin. However, the reticulated fibril ratio was dramatically increased in cells which were transfected with PSTPIP1-A230T and wild type pyrin, which corroborates earlier reports that mutant forms of PSTPIP1 bind to Pyrin with higher affinity [Shoham et al., 2003; Yu et al., 2007].

There are several potential explanations for the increase in reticulated fibril formation in the presence of mutant PSTPIP1. The first possibility is that mutant PSTPIP1 binds more tightly to Pyrin and they may form more reticulated fibrils together. For this reason, colchicine may not disrupt the pyrin-PSTPIP1 interaction sufficiently to prevent or delay reticulated fibril formation. Secondly, PSTPIP1-A230T mutant protein may take a critically different conformation when compared to wild type PSTPIP1 which would prevent a possible direct interaction between colchicine and PSTPIP1. Lastly and less possibly, colchicine may directly interact with other yet unidentified pyrin-interacting protein(s) which may facilitate reticulated fibril formation.

The effect of colchicine on the number of reticulated fibrils in the presence of mutant PSTPIP1 is still unknown. It was demonstrated previously that PSTPIP1 is involved in cell migration [Cortesio et al., 2010]. Certainly investigating the clinical significance and the biological impact of pyrin-PSTPIP1 interaction on cell functions remains unanswered.

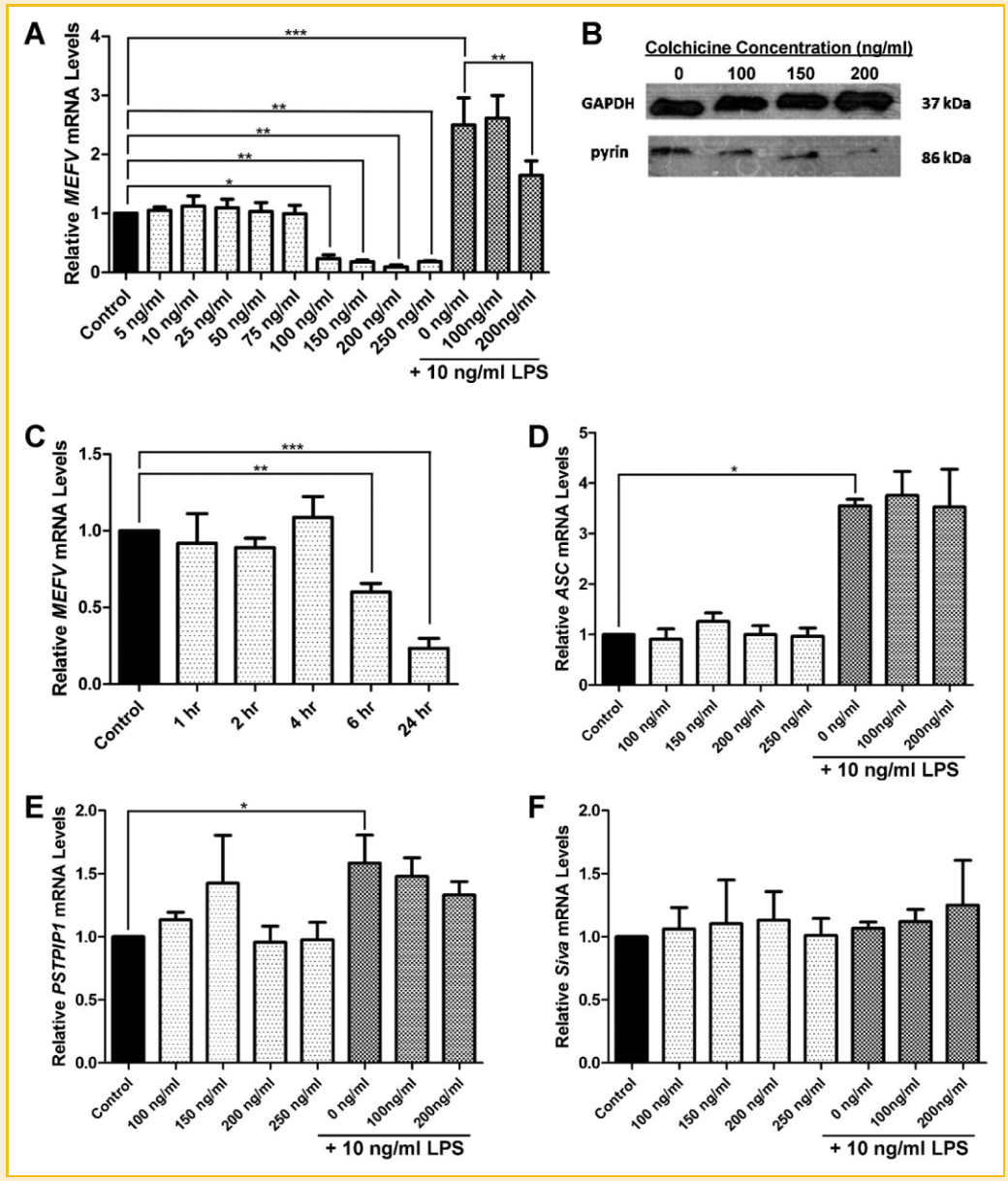

Fig. 6. The expression analysis of pyrin and its interacting proteins in colchicine treated THP-1 cells. A,D,E,F: Shows the quantitative real-time PCR results for *MEFV*, *ASC*, *PSTPIP1*, and *Siva* mRNA products after THP-1 cells are cultured for 24 h exposed to different colchicine concentrations, normalized to the housekeeping *ACTB*, respectively. Western Blot analysis for pyrin in THP-1 cells treated with different concentrations of colchicine for 24 h is shown in (B). C: Shows *MEFV* expression levels in THP-1 cells exposed to 100 ng/ml colchicine at different time points. Error bars indicate standard deviations. The statistically significant differences are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Another important structure is the ASC speck, which resembles the pyroptosome. Colchicine decreases ASC speck formation in the absence of pyrin in a concentration that does not affect the gross microtubular architecture. This implies that the effect of colchicine may be through some other mechanism that does not involve microtubules. It has been previously shown that in transfected HeLa cells, ASC speck formation is likely due to its diffusion rather than its interaction with other proteins according to computational models drawn through real time kinetic analysis of speck formation [Cheng et al., 2010]. This leads to the speculation that the decrease in ASC speck formation may be the direct result of an interference of colchicine on ASC polymerization. On the other hand, even if ASC speck formation occurs via microtubules, in order to disrupt the interaction of ASC and microtubules, microtubule depolymerization is not necessary.

Another important element to consider is that the colchicine's speck-disrupting effect is not seen in cells expressing wild-type pyrin, but only in M694V, M680I, and V726A pyrin-expressing cells. However, these three mutations promote speck formation further compared to wild type Pyrin in the absence of colchicine. So, colchicine acts as to bring down the speck formation rates to those of wild type pyrin in these mutants. This may be the main mechanism

how FMF patients benefit from colchicine. The increased tendency of pyroptosis initiation and inflammasome activation through ASC speck formation with disease-causing pyrin variants could be countered by non-microtubule depolymerizing concentrations of colchicine, and this would decrease the rate, severity, and consequences of inflammatory attacks in FMF patients. In particular, the increased tendency of HeLa and COS7 cells to undergo cell death while over-expressing ASC for long periods of time is important in the sense that ASC is the only known initiator of pyroptosis [Yu et al., 2007]. The severe FMF phenotype associated with M694V homozygotes and the increase in speck formation in M694V-expressing HeLa cells observed in our experiments also support this idea. Although the mechanism of action of colchicine proposed here requires further validation by in vivo experiments, these findings are very promising for elucidating FMF pathogenesis and colchicine action.

Regulation of *MEFV* expression in response to colchicine treatment has been investigated previously. One study demonstrated that *MEFV* is up-regulated in response to colchicine in monocytes [Centola et al., 2000]. On the other hand, it is repressed in monocytes by the anti-inflammatory cytokines. In neutrophils, colchicine and LPS had no effect while IFN-γ causes an increase in *MEFV* expression. Furthermore, another group showed reduced *MEFV* levels in FMF patients (59% of these patients received colchicine) [Notarnicola et al., 2002]. Also, in the same study, there were no differences between colchicine treated and non-treated patients in terms of *MEFV* levels.

Based on the hypothesis that colchicine may have a specific effect on *MEFV* expression, differentiated THP-1 cells were exposed to different concentrations of colchicine. qRT-PCR and western blot studies revealed that colchicine reduces MEFV expression compared to control cells. These results stand in contrast to previous studies in terms of MEFV expression [Centola et al., 2000; Notarnicola et al., 2002]. This is likely due to the fact that firstly, we have determined colchicine concentration after careful titration by observing microtubule architecture, and could be seeing more specific effects of colchicine on cellular level. Secondly, we used PMA-differentiated THP-1 cells instead of primary monocytes/netrophils or whole blood cells of FMF patients. In patients, the isolated cells are (a) exposed chronically rather than acutely to colchicine and (b) the isolated cell population that is tested is often a mixed population of mature and immature cells whereas the differentiated cell line is expected to respond more uniformly. *MEFV* and *ASC* were up-regulated by LPS in our experiments, similar to previous studies [Centola et al., 2000; Masumoto et al., 2006]. We also found that *PSTPIP1* expression was increased by LPS. This finding lends additional support to the idea of co-action of these three proteins in regulating innate immunity.

How colchicine down-regulates *MEFV* expression in THP-1 cells and what clinical significance this finding holds remains to be answered. Since, in our experiments stress fibers and shorter filopodia were observed in colchicine treated THP-1 cells, we suggest that colchicine-induced effects on actin re-organization may reduce *MEFV* levels. Colchicine seems to cause actin re-organization while microtubule structure is intact. As previously demonstrated, pyrin is an actin binding protein that is found specifically in polymerizing actin filaments [Waite et al., 2009b]. Pyrin, or *MEFV* transcription, may also be affected by this re-organization of the actin cytoskeleton, which may explain why reduced *MEFV* levels were observed after colchicine administration. The data presented here reveal an important connection between colchicine and *MEFV* transcription, which has potential to explain the remarkable efficacy of colchicine in preventing FMF attacks by reducing the mutant protein level in FMF patients. Therefore, our data provide evidence for a pro-inflammatory role of pyrin, suggesting that FMF may result from gain of function mutations.

Here, we have clarified the effect of colchicine on pyrin and its interacting partners on both a cell biology and expression level. It has been thought that inhibition of microtubule polymerization is the main mechanism of action of colchicine. However, our results indicate that further exploration into the exact cellular mechanism explaining the efficacy of colchicine in suppressing FMF attacks is warranted.

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