Abstract

Objective
Aberrant PYRIN inflammasome activity triggers FMF pathogenesis but the exact mechanism remains elusive and an obstacle to efficient treatment. Herein, we sought to identify PYRIN inflammasome specific mechanisms to improve FMF treatment and diagnostics in the future.

Methods

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PYRIN-specific protein secretion was assessed by proteome analysis in U937 derived macrophages, and specific findings were confirmed in PYRIN inflammasome activated monocytes from healthy blood donors (HD) and FMF patients, stratified by MEFV genotype categories corresponding to a suspected increasing FMF disease severity.

**Results**

Proteome data revealed differential secretion pattern of IL1Rα from PYRIN and NLRP3 activated U937 derived macrophages, which was verified by ELISA and qPCR. Moreover, PYRIN activation significantly reduced IL1RN mRNA expression (p<0.001) and IL1Rα secretion (p<0.01) in healthy donor- and FMF monocytes, respectively. Independent of MEFV genotype, unstimulated FMF monocytes from colchicine treated patients secreted lower amounts of IL1Rα as compared to healthy donors (p<0.05) and displayed decreased ratios of IL1Rα/IL1β (p<0.05), suggesting a reduced anti-inflammatory capacity.

**Conclusion**

Our data show an inherent lack of IL-1 receptor antagonist expression specific to PYRIN inflammasome activation, providing a new mechanism underlying FMF pathogenesis. The reduced IL1Rα levels in FMF monocytes suggest a diminished anti-inflammatory capacity potentially leaving FMF patient monocytes more sensitive to pro-inflammatory stimuli, regardless of being in colchicine therapy. Thus, considering the potential clinical consequence of reduced monocyte IL1Rα secretion in FMF patients, we suggest further investigations into IL1Rα dynamics and its potential implications for FMF treatment in the future.

**Keywords**

Autoinflammation/FMF/IL1Ra/MEFV/PYRIN

**Introduction**

The PYRIN inflammasome was recently discovered as an intracellular indirect pattern recognition receptor of the innate immune system. It is a new member of the inflammasome macromolecular complex family that initiates pro-inflammatory responses by activating Caspase-1 (CASP1) dependent Interleukin-1β (IL1B) processing [1, 2]. PYRIN inflammasome assembly is facilitated by PYRIN that has been activated by bacterial modifications of RhoA activity [2] through downstream effector kinase release of inhibitory PYRIN phosphorylation [1]. PYRIN is encoded by the MEFV (Mediterranean Fever) gene and located on chromosome 16 in humans. In 1997, mutations in the

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MEFV gene were identified and associated with the autoinflammatory disease, Familial Mediterranean fever (FMF) [3, 4]. As a monogenic hereditary disease, FMF is characterized by periodic attacks of fever accompanied by varying degrees of serositis, myalgia, skin involvement, and elevated levels of acute phase reactants [5, 6]. It mainly affects people of Mediterranean origin with a reported prevalence in the range of 1:400 – 1:1000 [7, 8]. Yet, with increasing rates of immigrants and refugees coming from high prevalent areas [9], FMF prevalence is expected to increase in low prevalent areas. Even though the FMF diagnosis is mainly established from clinical symptoms, diagnostics and disease monitoring is highly dependent on genetic screening for MEFV variants and assessment of unspecific acute phase reactants such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and serum amyloid A (SAA) in patient blood. In addition, clinical symptoms are heterogeneous and many FMF patients, including apparently healthy carriers of low-penetrant MEFV variants, exhibit varying degrees of sub-clinical inflammation [10, 11].

Colchicine, the standard treatment for FMF patients [12], reduces the number and severity of attacks, the prevalence of chronic subclinical inflammation, amyloidosis, and associated kidney failure among others [10, 12], yet the underlying mechanism of action is unknown. However, treatment is still not curative, patients with severe disease often experience treatment resistance [13, 14], and the maximum tolerated colchicine dose is not always adequate to control FMF activity [15]. As in a few other auto-inflammatory disorders [16], non-responder FMF patients may alternatively be treated with IL-1 receptor antagonists- (IL1Ra) or IL-1 antibodies to relieve symptoms [17-20]. Thus, the limited knowledge of how MEFV variant properties affect FMF pathogenesis and shape disease phenotype, hinders effective treatment of this increasing population of patients. We therefore set out to investigate macrophage PYRIN inflammasome activation to gain more mechanistic insight to FMF pathogenesis that may open up new alleys for improving the treatment of FMF patients.

**Materials and Methods**

**U937 derived macrophages**

The U937 line (ATCC CRL-1593.2) was maintained in medium consisting of Roswell Park Memorial Institute (RPMI 1640 ATCC modified formulation; A1049101, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS)(16140071, ThermoFisher Scientific) and 1% penicillin-streptomycin (PS)(15140122, ThermoFisher Scientific) at 37°C. For differentiation, cells were plated at 50.000 cells/cm² and treated with 100 ng/mL Phorbol 12-myristate 13-acetate.
(PMA)(P1585, Sigma-Aldrich) for 72h in ECM-coated (E1270, Sigma-Aldrich) culture plates, followed by a resting period of 72h in PMA-free complete culture medium. Optimal conditions for differentiation were established prior to study execution (Data not shown). Differentiation was verified by morphological changes and increased mRNA expression of macrophage markers; CD11b, CD14, CD86 and HLA-DRB1 (Supplemental Figure S1).

Inflammasome activation in U937 derived macrophages
Inflammasome activation in macrophages was obtained by priming for 4 hours with 1 µg/mL ultrapure E.coli LPS (tlrl-3pelps, InvivoGen) followed by an activation step of; 1 µg/mL C3 toxin (CT04-B, Cytoskeleton) (denoted LPS/C3) for PYRIN activation, 2,5 mM ATP (A6419, Sigma-Aldrich) (denoted LPS/ATP) for NLRP3 activation, and no stimuli for LPS induced effects (denoted LPS/-). All activation steps were conducted in serum free media for 16 hours. Unprimed cells in serum free medium were included as unstimulated controls (denoted -/-). Inflammasome activation was performed in 4 biological replicates according to previously described protocols [1, 21] and preliminary model setup experiments with varying concentrations of activators and time periods (Supplemental Figure S2B-E). Cell supernatants were collected, cleared by centrifugation and stored at -30°C until protein quantification. Cells were detached by incubation in PBS at 37°C, counted, lyzed in RNeasy mini kit lysis buffer (RLT) and stored at -80°C until RNA extraction.

Human plasma samples and primary isolated monocytes
Patient and donor blood samples were collected from healthy blood donors (HD) and FMF patients upon signing written consent. Patients were stratified in three MEFV genotypes groups (Supplementary Table S2); FMF group 1) patients with MEFV variants classified as variants of unknown significance or no detected variants (n=10), FMF group 2) patients with variants classified as pathogenic or likely pathogenic in mono-allelic form (n=7), and FMF group 3) patients with variants classified as pathogenic or likely pathogenic in bi-allelic form (n=6). The study protocol was approved by The Regional Committees on Health Research Ethics for Southern Denmark (S-20170020). Blood was collected in; Plasma Separation tubes (368270, Becton Dickinson), and CPD tubes (455056, Greiner Bio-One) for PBMC isolation. Plasma samples were centrifuged at 1500 x g for 10 min and stored at -80°C, and PBMCs were isolated from CPD samples by density gradient centrifugation of whole blood, using Ficoll-Paque Plus (17144002, GE Healthcare) in Sepmate separation tubes (85450, Stemcell Technologies). Cells were processed within 24 hours after blood
sampling and PBMCs stored in liquid nitrogen until use. Monocytes were enriched from thawed PBMCs by negative selection using the EasySep enrichment kit without CD16 depletion (19058, Stemcell Technologies) according to manufacturer’s recommendations.

**Inflammasome activation in primary isolated human monocytes**

PYRIN inflammasome activation was obtained by addition of 10 ng/mL ultra-pure *E.coli* LPS (tlrl-3pelps, InvivoGen) and 1 µg/mL C3 toxin (CT04-B, Cytoskeleton) in serum reduced media, Optimem (31985062, ThermoFisher Scientific) for 16 hours. Monocytes with 10 ng/mL LPS addition or no stimuli for 16 hours in Optimem were included as LPS- and baseline controls, respectively. For PPARα activation, PPARα agonist WY14643 (C7081, Merck) or vehicle (DMSO) was added at 50 ng/mL for 16 hours together with LPS (10ng/mL) and C3 (1 µg/mL). Cell supernatant was removed, cleared by centrifugation and stored at -30°C until protein quantifications. Cells were lysed by addition of TRI Reagent (AM9738, ThermoFisher Scientific) and cell lysate was stored at -80°C until RNA extraction.

**RNA extraction and relative quantitative qPCR**

Total RNA was extracted from U937 derived macrophages using the RNeasy mini kit (74104, Qiagen) or from primary isolated monocytes using TRI Reagent (AM9738, ThermoFisher Scientific) according to the manufacturer’s instructions. RNA concentrations were assessed by Nanodrop. For cDNA synthesis, 0.2-0.4 µg of total RNA was reverse transcribed using High Capacity cDNA RT kit (4368813, ThermoFisher Scientific), and quantitative real-time PCR (qRT-PCR) was performed in technical triplicates with 2 ng of cDNA and 3 pM of forward and reverse primer in a 10 µl reaction mixture using Power SYBRGreen mastermix (4367659, ThermoFisher Scientific). Primers were designed to preferably span exon-exon junctions using the NCBI Primer-Blast software [22]. Primer sequences are listed in Supplemental Table S1. PCR was run on a QuantStudio 7 Flex (4485698, ThermoFisher Scientific) or LightCycler 480 II (05015278001, Roche), using the following PCR conditions: 95 °C for 10 min prior to 45 PCR cycles of: denaturation at 94°C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Robust and valid qRT-PCR data were obtained by normalizing raw data against multiple stably expressed endogenous control genes as determined by the geNorm algorithm in qbase+ version 3.2 (Biogazelle, Zwijnaarde, Belgium)[23].

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Measurements of cytokines and proteasome subunits in cell supernatant and plasma samples

Quantifications of cytokines in cell media supernatants and human plasma were performed using a custom designed Luminex Magnetic assay (LXSAHM-04, R&D systems), by Uplex Assay (K15067L-1, Meso Scale Diagnostics), or by individual ELISA kits; human IL-1beta/IL-1F2 (DLB50), according to manufacturer’s instructions.

Proteome analysis

Proteins were isolated from cell media supernatant of inflammasome activated U937 derived macrophages as described above (n=4), by acetone precipitation. The protein pellet was re-dissolved in 0.2 M TEAB tetraethylammonium bicarbonate (TEAB) followed by DTT reduction (5mM, 50C, 30 min), IAA alkylation (15 mM, RT, 30 min) and proteolysis using 1 µg trypsin. The resulting tryptic digests were labelled with isobaric tags using the tandem mass tag (TMT) 10plex reagent according to manufacturer’s recommendations (90110, ThermoFisher Scientific). Each sample set was fractionated into 24 fractions using hydrophilic interaction liquid chromatography (HILIC) fractionation and analyzed by tandem mass spectrometry (nano-HPLC MSMS) as previously described [24], with the following modifications; peptide mixtures were separated using a 30 min linear gradient from 95% A (0.1% formic acid) to 30% B (100% acetonitrile), and maximum ion injection time for MSMS analysis was set to 500 ms. Raw data were processed using the Proteome Discoverer 2.1.0.81 software integrated with the MASCOT (version 2.4) and the SEQUEST database search program [25]. The search parameters were set to: MS accuracy 8 ppm, MSMS accuracy 0.05 Da with two missed cleavages allowed, fixed modifications of N-terminal, lysine (both 10-plex TMT), and cysteine blocked with carbamidomethyl, and variable modification; methionine oxidation, and deamidated asparagines and glutamines. Tandem mass spectra were searched against the Swissprot database restricted to humans. Proteins were inferred on the basis of at least one unique peptide identified with high confidence (false discovery rate < 1%). False discovery rates (FDRs) were obtained using the Percolator selecting identification with a q value of 0.01 or less. The TMT quantification was performed using Proteome Discoverer with reporter ion area integration within a 20-ppm window and protein abundances were relative to a pool of all samples.

Proteome data analysis

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Log2 fold ratios for each treatment vs. control (LPS/- vs. control, LPS/C3 vs. control and LPS/ATP vs. control) were calculated from mean relative abundance of each identified protein. Keratins are well-known contaminants in mass-spectrometry and were excluded from the analysis. Based on DAVID Functional Annotation Table for GO (gene ontology) term Cellular Compartment analysis (Bioinformatic Database version 6.8) [26, 27], to avoid identification of contaminating unspecific intracellulary associated proteins, we selected only terms that referred to the extracellular space and cell surface (GO:1903561~extracellular vesicle, GO:0070062~extracellular exosome, GO:0043230~extracellular organelle, GO:0044421~extracellular region part, GO:0005615~extracellular space, GO:0019898~extrinsic component of membrane, GO:0009986~cell surface, GO:0044420~extracellular matrix component, GO:0005578~proteinaceous extracellular matrix, GO:0097610~cell surface furrow).

Up- and down regulated proteins were defined as Log2 fold change >0.1 or <0.1, respectively. Lists of regulated proteins (treatment vs. control) were uploaded to the functional annotation tool, DAVID (Bioinformatic Database version 6.8) [26, 27] for GO (Gene Ontology) enrichment analysis. Significance was tested by modified Fishers Exact test with false discovery rate (FDR) correction and FDR<0.05 was considered significant. PYRIN inflammasome (C3/LPS) specific proteins were presented according to their level of up- or downregulation compared to LPS, after subtracting proteins similarly regulated by NLRP3 inflammasome (LPS/ATP) activation.

Statistical analysis

Statistical significance of the difference between means was determined by two-tailed t-test, or by one- or two-way ANOVA followed by post-hoc tests as indicated in the figure legends for each experiment. Repeated measures tests were employed for analysis of HD and FMF monocyte gene- and protein expression upon inflammasome activation. A mixed effect model were used instead of repeated measures when baseline values were below detection levels. Values of relative normalized gene expression and protein concentrations in cell supernatants were presented as means with SD including individual values. Monocytes displaying a “pre-activated” profile were excluded from experiments and are not included in the presented data (HD n=1, FMF n=2). Pre-activation was defined as; (1) Baseline IL1B mRNA normalized relative quantity above 1000 (scaled to the sample with lowest expression using qBase+ software), or (2) IL1β protein concentration in cell supernatant above 1000 pg/mL at baseline. Effect of PPARα agonist, WY14643 treatment was presented as means and SD of individual expression ratios to DMSO (vehicle), including individual
values. Three observations were missing from the PPARα agonist experiment due to failed measures of relative normalized gene expression or protein in cell supernatant. Sample size for each experiment is presented in each figure. For cell line experiments, n denotes the number of performed experiments with cell cultures separated by at least 3 passages, and for experiments involving healthy donor and FMF patient plasma or monocytes, n denotes the number of individuals. Continuous variables (cytokine mRNA and protein expression) from patient- and healthy donor data were log-transformed to obtain Gaussian distribution and fulfill the requirements for two-way ANOVA statistics prior to statistical analysis, when this was required. GraphPad Prism (version 7.04) was used for statistical analysis and significance of enrichment analysis was tested by modified Fishers Exact test with false discovery rate (FDR) correction by built-inn tests in the DAVID tool.

Results
PYRIN inflammasome activation in U937 derived macrophages impede IL-1 receptor antagonist expression

With the intent of mimicking FMF pathogenesis, we differentiated the human pro-monocytic cell line, U937 to macrophages (Supplemental Figure 1A-E) and primed with LPS to induce pro-inflammatory gene expression [28] prior to specific inflammasome activation (Supplemental Figure 1F and 2B). We designed the model with the intent of discriminating effects derived from LPS-induced TLR4-mediated cell activation (LPS treated cells) [29], effects derived from its potential LPS induced cross-activation of NLRP3 inflammasome (LPS/ATP treated cells) [30] and PYRIN inflammasome activation (LPS/C3 treated cells) [1] (Figure 1A). As expected, LPS priming elicited by itself a strong pro-inflammatory transcriptional response including increased expression of IL1β (Supplemental Figure 1F) as well as cell swelling and loss of membrane integrity (Supplemental Figure 2B-E). This inflammatory phenotype of the U937 derived macrophages was exaggerated by ATP and C3 addition in a dose- and time dependent manner in our initial model setup experiments (Supplemental Figure 2C-E), and thus together confirmed our in vitro design.

We next used mass spectrometry of media supernatants from stimulated U937 derived macrophages (n=4) to specifically identify molecular networks that distinguish between LPS-induced cell activation, NLRP3 inflammasome (LPS/ATP), and PYRIN-inflammasome (LPS/C3) signaling. Initially, the pro-inflammatory response of U937 derived macrophages was confirmed by measurements of IL1β and TNFα in cell supernatants (Figure 1B). Using mass spectrometry data
we identified a total of 1556 robustly present proteins in the media, and Gene Ontology (GO) enrichment analysis of the upregulated proteins for the three subsets (101, 434 and 344 for LPS, NLRP3- and PYRIN inflammasome respectively) as compared to control (non-stimulated) revealed enrichment (FDR<0.05) of biological processes related to neutrophil chemotaxis, inflammatory response, and positive regulation of inflammatory response for LPS stimulated U937 derived macrophages (Supplemental Figure 3A). Further stimulation of PYRIN- or NLRP3 inflammasome with C3 or ATP, revealed 18 and 12 annotated biological processes, respectively (Supplemental Figure 3B-C), three of which were shared and some that remained specific to each stimulation. This confirmed the onset of LPS induced inflammatory effects [31, 32], and that the underlying mode of action for NLRP3 inflammasome, and PYRIN inflammasome activation clearly differs, in support of our design.

We next identified mechanisms involving secretion of proteins specific to PYRIN inflammasome activation and FMF disease by conducting cell compartment analysis, classifying 924 proteins as secreted or associated with the cell surface. As such, we identified 86 upregulated and 179 downregulated proteins unique to PYRIN inflammasome activation (Figure 1C). Interestingly, by listing and evaluating the top 10 up-and down regulated PYRIN specific proteins (Figure 1C) we recognized the IL-1 receptor antagonist (IL1Ra), a key regulator of IL-1 signaling used for treating other immune affected patients and FMF colchicine non-responders [17-19, 33]. We therefore decided to analyze in detail, whether IL1Ra-dependent mechanisms are specifically associated to the FMF phenotype.

**IL-1 receptor antagonist expression is abrogated by PYRIN inflammasome activation and compromised in unstimulated FMF monocytes**

Initially, we verified the mass spectrometry data on differential IL1Rα abundance in PYRIN- and NLRP3 inflammasome activated U937 derived macrophage medium supernatants, and found that an increase in IL1RN mRNA expression specifically was associated with NLRP3 mediated inflammasome activation, but lacking for PYRIN inflammasome activation (Figure 1D). In agreement with this finding, the LPS induced IL1Rα protein expression was amplified from 13866±2913 to 21265±3650 pg/mL (mean, SD, n=4, p<0.001) by NLRP3 activation, whereas IL1Rα remained unaltered at 11042±1171 pg/mL in PYRIN-stimulated U937 derived macrophages (Figure 1E). This may indicate an inherent absence of IL1Rα dampening of IL-1 signaling in the monocyte cell lineage of FMF patients, which potentially exaggerate the FMF disease. Monocytes...
were therefore isolated from FMF patients presenting bi-allelic, pathogenic exon 10 MEFV variants and healthy blood donors (Figure 2A) (Supplemental Table S2). Both LPS activation - and PYRIN inflammasome activation induced a substantial pro-inflammatory response measured by IL1β and TNFα protein secretion (Figure 2B-C), however only IL1β levels were significantly affected by additional PYRIN inflammasome activation, indicating that TNF expression is mainly controlled by LPS activation of the TLR4 receptor while IL1B expression is further triggered by PYRIN inflammasome activation [34, 35]. The magnitude of the pro-inflammatory response was not affected by FMF disease under the present experimental conditions (Figure 2B-C). Confirming our U937 cell model derived data (Figure 1), we found a substantial increase in IL1Ra for both FMF patients and healthy donor monocytes for LPS treatment (Figure 2D-E), while PYRIN inflammasome activation abrogated the IL1Ra expression, with IL1RN mRNA levels declining below baseline, from 14.3±4.1 to 6.2±2.1 (mean±SD, n=6, p<0.001) in healthy donors and from 11.6±5.7 to 3.7 (mean±SD, n=6, p<0.001) in FMF patients, compared to LPS treated monocytes (Figure 2D). This was supported by a reduction in cell supernatant IL1Ra from 8521±3733 pg/mL to 3143±1742 pg/mL (mean±SD, n=6, p<0.01) in healthy donors and from 7761±7423 pg/mL to 1549±1248 pg/mL (mean±SD, n=6, p<0.01) in FMF patients compared to LPS treated monocytes (Figure 2E). IL1Ra levels in plasma did not show any robust changes between FMF patients and healthy donors. Instead, elevated plasma IL1Ra levels to some degree seemed to coincide with the presence of an ongoing FMF attack (Figure 2F), which is in line with IL1Ra also being a liver secreted acute phase reactant [36]. To delineate the underlying mechanism of macrophages’ and monocytes’ inability to mount an appropriate IL1Ra response upon PYRIN inflammasome activation, we investigated the nuclear receptor PPARα:RXRα complex. It is known that the Retinoic acid receptor RXR-alpha (RXRα) facilitates Peroxisome proliferator-activated receptor alpha (PPARα)-mediated IL1RN transcription from the IL1RN promotor [37-39], thus we sought to investigate whether IL1RN transcription and expression could be rescued by addition of the PPARα agonist WY14643 to PYRIN-activated monocytes (Figure 3A). Contrary to our expectations, we found that while PPARα agonist treatment failed to induce or rescue the abrogated IL1RN transcription in PYRIN activated monocytes (p<0.001) (Figure 3B-top), it did confer an inhibitory effect on the pro-inflammatory IL1B mRNA transcription in both healthy donor and FMF monocytes (p<0.001) (Figure 3B-bottom). This was confirmed at the protein level, where secreted IL1Ra and IL1β were reduced by PPARα agonist treatment (p<0.01) (Figure 3B-C), suggesting that WY14643 successfully agonized the RXRα independent trans-repressing PPARα effect, while
failing to agonize the RXRα dependent trans-activating effect of PPARα (Figure 3D). Accordingly, PPARA mRNA expression increased in patient monocytes upon PYRIN inflammasome activation (p<0.05) (Figure 3E) and RXRA mRNA expression declined, however only significantly (p<0.05) in healthy donor monocytes as compared to LPS treatment (Figure 3F). Thus, our data indicate a specific failure in monocytes to mount an appropriate IL1Ra response upon PYRIN inflammasome activation, which at least partly could be mediated by an RXRα dependent anti-inflammatory mechanism of PPARα. However, the exact mechanism and its implications for IL1Ra dynamics in FMF patients would have to be further investigated. We then finally tested IL1Ra release from baseline-, LPS- and PYRIN inflammasome activated monocytes from a larger group of colchicine-treated, attack-free FMF patients, representing a more diverse variety of MEFV variants, grouped according to the expected clinical significance (Supplemental Table S2). Monocytes were isolated and stimulated as described above (Figure 2A) and cell supernatant IL1Ra and IL1β concentrations were assessed at baseline and upon LPS- and PYRIN-inflammasome activation, respectively. In line with our above findings, PYRIN inflammasome activation reduced IL1Ra secretion below baseline levels, however now also with a statistical significant difference between HD- and FMF monocytes (p<0.05) (Figure 4A). In addition, we found that non-stimulated (baseline) FMF patient monocytes released significantly lower amounts of IL1Ra (1347±1159 pg/mL, n=23) extracellularly than healthy donors (3049±1728 pg/mL, n=6) (p<0.05) (Figure 4A). Yet, there was no difference in secretion levels of IL1Ra (p>0.7556) nor in IL1Ra/IL1β ratio (p>0.3746) levels between the three different FMF groups (Supplemental Table S2), either at baseline or from LPS- or LPS/C3 treatment (Data not shown) (Figure 4A-B). An IL1Ra/IL1β ratio above 100 is considered to reflect an anti-inflammatory functional phenotype [40, 41]. In our FMF patient monocyte, IL1Ra/IL1β ratios were just balancing around this threshold (121.5±92.0, n=23) whereas healthy donor monocytes exhibited a clear anti-inflammatory phenotype (255.0±138.5, n=6) (Figure 4B). As expected, LPS- and PYRIN inflammasome activation caused an overall reduction of the IL1Ra/IL1β ratio, however maintaining the significant difference between HD- and FMF monocytes (p<0.05) (Figure 4B). These low IL1Ra and IL1Ra/IL1β ratio baseline conditions in FMF patient monocytes likely imply a more sensitive monocyte phenotype with reduced capacity to control and shut down potential pro-inflammatory responses, thereby triggering the FMF disease attacks.

Discussion

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Deficiency of PYRIN inflammasome assembly and regulation due to MEFV mutations is the most recently suggested molecular explanation of FMF pathogenesis, but many questions regarding the specific mechanism by which mutational altered PYRIN proteins confer the pro-inflammatory phenotype in FMF patients remain to be answered. Herein, we demonstrate how activation of the PYRIN inflammasome, contrary to LPS- and NLRP3 inflammasome activation, abrogates IL1Rα expression, suggesting a PYRIN inflammasome specific deregulation of IL-1 signaling (Figure 4C). Together with the reduced IL1Rα levels and Il1Rα/IL1β ratios in unstimulated FMF monocytes, these findings calls for a further investigation into how this may have translational potential for treating FMF patients. While colchicine is effective in reducing symptoms in most patients IL1Rα therapy in addition to colchicine may augment the abnormally reduced IL1Rα-mediated anti-inflammatory capacity of FMF patients monocytes, and hereby potentially provide substantial relief of symptoms, especially relevant for certain groups of FMF patients where standard colchicine treatment is insufficient to control symptoms.

Thus, whereas previous finding have shown a delayed IL1Rα response in TLR stimulated monocytes from patients with the auto-inflammatory disease, cryopyrin-associated periodic syndrome (CAPS) [42], our direct finding of decreased steady-state anti-inflammatory capacity in FMF patient monocytes with an absence of an IL1Rα response upon PYRIN inflammasome activation, is to the best of our knowledge novel. IL-1 signaling has recently been linked to the induction of trained immunity in myeloid cells by introducing epigenetic changes that increase monocyte and macrophage responsiveness to pro-inflammatory stimuli [43, 44]. Interestingly, IL1Rα treatment has proven important for reversing this process in primary human monocytes [45] and in hematopoietic mouse stem cells [43]. When lacking the IL-1 dampening capacity of IL1Rα in FMF monocytes as we describe here, it is likely that even minute amounts of microbial constituents (PAMPs) or other fluctuations in cell homeostasis (HAMPs) may shift the anti-inflammatory/inflammatory balance (Figure 4C). As such, FMF monocytes may upon every disease attack be shifted to the modified “trained” state [44], exhibiting an exaggerated pro-inflammatory phenotype. However, whether the decreased IL1Rα secretion in monocytes as observed in this study, leads to consecutive aggravated immune de-regulation in FMF patients, remains to be further addressed. The efficacy of IL1-receptor antagonists for certain subgroups of FMF patients has been confirmed, where IL-1 antagonists in FMF patients unresponsive to colchicine, reduces the number of attacks and the levels of acute phase reactants [18, 46, 47]. Moreover, IL-1 antagonists have been shown to lower proteinuria in patients with complicating AA amyloidosis [48-50]. Thus, our data
suggest further investigations of the clinical relevance of monocyte IL1Rα secretion in FMF pathology, considering the potential beneficial effects of extending IL-1 antagonist therapy to a broader spectrum of FMF patients. Since our data shows that FMF patients, independent of MEFV genotypes and despite of being in colchicine treatment, have compromised IL1Rα monocyte capacity, all FMF patients may likely benefit from IL-1 antagonist treatment in combination with colchicine. This is in agreement with a very recent hypothesis proposed during the finalization of our study, that IL-1 blockade should be considered as first line treatment to prevent irreversible loss of beneficial effects in naïve patients with AA amyloidosis, proteinuria or renal impairment [51]. Also our data suggest that measuring baseline IL1Rα secretion or IL1Rα/IL1β balance in patient monocytes may serve as a new obvious clinical tool for monitoring disease activity and treatment progress. Yet, since we focused on circulatory monocytes in patients, we cannot exclude that IL1Rα dynamics in resident macrophages or in-fluxing neutrophils to serosal fluids may behave differently. Also, whether other treatment schedules can be developed from our new knowledge has to be further investigated. For instance, it is known that PPARα exerts its anti-inflammatory effect by two different routes; by heterodimerizing with RXRα in the PPARα:αRXRα complex to induce IL1RN transcription through binding to Peroxisome-Proliferator Response Element (PPRE) in the promotor of IL1RN [37] and by trans-repressing of pro-inflammatory gene transcription through inhibitory binding of transcription factors AP-1 and NF-κB [52]. Herein, agonizing PPARα:RXRα activity did not induce IL1RN transcription, while RXRα independent trans-repression of IL1B was successfully achieved. Recent knowledge [53, 54] suggests that PPARα and RXRα also play key roles in bridging metabolism and inflammation through lipid activation, which potentially may explain part of the observed FMF phenotype heterogeneity. Together this could suggest that dietary restrictions may benefit FMF patients.

The limitation of the study include absence of MEFV variant-matched non-colchicine treated patients to investigate if the low IL1Rα levels are caused by colchicine treatment. However, whether or not this is colchicine induced, it is likely disadvantageous for the anti-inflammatory capacity of FMF patient monocytes.

In conclusion, our data collectively points to aberrant IL1Rα dynamics underlying FMF pathogenesis, and we therefore propose further investigation into IL1Rα dynamics in FMF patients and its implications for developing new treatment schedules for patients with poor responsiveness or tolerance to colchicine.
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Author contributions

SBM: Study concept and experiment design, patient enrollment and sample collection, experiment execution, data analysis and interpretation, manuscript writing and figure preparation, manuscript editing and final approval, funding; KLL, EHB and HCB: Experiment execution, data analysis, manuscript editing and final approval; TM, ABH: Patient enrollment and sample collection, manuscript editing and final approval; MAJ: Experiment design, critical discussion, manuscript editing and final approval; ISJ: Study concept and experiment design, patient enrollment and sample collection, critical discussion, manuscript editing and final approval, funding; DCA: Study concept and experiment design, data analysis and interpretation, critical discussion, manuscript writing and figure preparation, manuscript editing and final approval.

Declaration of interest

The authors declare no competing interests.

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Figure Legends

Figure 1. PYRIN- and NLRP3 inflammasome activation in U937 derived macrophages exerts differential effects on IL1Rα expression.

(A) Overview of inflammasome activation model design, with differentiation between LPS derived effects (LPS), NLRP3- (LPS/ATP)-, and PYRIN- (LPS/C3) inflammasome activation respectively. Dashed lines indicates the potential cross activating effects of LPS on NLRP3 inflammasome activity. (B) IL1β and TNFα in cell supernatant of U937 derived macrophages upon LPS priming and inflammasome activation (n=4). (C) Venn diagram of up (Log2FC>0.1) - and down

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(Log$_2$FC<0.1) regulated proteins in cell supernatant of PYRIN- and NLRP3 inflammasome activated cells as compared to LPS treated cells, and top 10 PYRIN specific up- and down regulated proteins ranked according to Log$_2$FC. (D-E) IL1RN mRNA expression (D) and protein release (E) of U937 derived macrophages upon LPS priming, and inflammasome activation (ATP and C3). LPS priming significance was tested by two-sample t-test and significance of PYRIN- (LPS/C3) and NLRP3 (LPS/ATP) inflammasome activation was tested compared to LPS treated cells for each time point by One-Way ANOVA. Correction for multiple comparisons was conducted using the Holm-Sidak method. * p<0.05; **p<0.01; ***p<0.001. # below assay detection limit

Figure 2. PYRIN inflammasome activation abrogate IL-1 receptor antagonist expression in primary isolated healthy donor and FMF patient monocytes.

(A) Experimental set-up; Healthy donor (HD)- and FMF patient monocytes were enriched from peripheral blood mononuclear cells (PBMCs) and stimulated with; no stimuli (baseline) LPS, or LPS/C3 (PYRIN) for 16 hours before harvest of cell supernatant and RNA. FMF patients were MEFV genotyped with bi-allelic pathogenic exon 10 variants (supplementary table S2). (B-C) Evaluation of IL1β (B) and TNFα (C) in cell supernatant to confirm the pro-inflammatory stimulation. (D-F) Evaluation of IL1RN mRNA expression (D) and IL1Rα secretion upon monocyte activation according to A, and (F) IL1Rα levels in plasma samples of the included healthy donors (HD) and FMF patients. Significance of LPS and PYRIN inflammasome activation (LPS/C3) was tested by repeated measures or mixed effect 2-Way Anova, followed by Holm-Sidak comparison of LPS vs. baseline and LPS/C3 vs. LPS for HD- and FMF monocytes respectively. Significance of IL1Rα plasma levels was tested by Mann Whitney test. ns = non significant, * p<0.05, ** p<0.01, ***p<0.001.

Figure 3. PPARα agonist WY14643 fail to rescue the abrogated IL-1 receptor antagonist expression in PYRIN inflammasome activated monocytes.

(A) Experimental set-up; PYRIN inflammasome activated (LPS/C3) healthy donor (HD)- and FMF monocytes was treated with PPARα agonist WY14643 or vehicle (DMSO) for 16 hours. FMF patients were MEFV genotyped with bi-allelic pathogenic exon 10 variants (supplementary table S2). (B-C) IL1RN and IL1B mRNA expression (B) and IL1Rα and IL1β protein secretion (C) upon
PPARα agonist treatment. Data are presented as the ratio to DMSO (vehicle) treatment. (D) Theoretical drawing of PPARα involvement in the regulation of anti- and pro-inflammatory gene transcription. (E-F) PPARA (E) and RXRA mRNA expression upon Pyrin inflammasome activation as described in Figure 2A. Significance of WY14643 treatment was tested by One-sample t-test comparing WY14643/DMSO ratio to a hypothetical mean of 1. Significance of PYRIN inflammasome activation (LPS/C3) on PPARA and RXRA was tested by repeated measures 2-Way Anova, followed by paired t-test comparison of LPS vs. LPS/C3 for HD- and FMF monocytes respectively. ns = non significant, * p<0.05, ** p<0.01, *** p<0.001.

Figure 4. Cell supernatant IL1Rα and IL1Rα/IL1β ratio is decreased in colchicine treated FMF patient monocytes compared to healthy donor monocytes. (A-B) Healthy donor (HD)- and FMF patient monocytes were enriched from PBMCs and stimulated as described in Figure 2A. FMF patients were grouped based on MEFV genotypes in; FMF 1) no variants or variants with uncertain significance (n= 10, green circles), FMF 2) mono-allelic pathogenic or likely pathogenic exon 10 variants (n=7, yellow circles), FMF 3) bi-allelic pathogenic or likely pathogenic exon 10 variants (n=6, blue circles). IL1Rα (A) and IL1Rα/IL1β ratio (B) was evaluated in the cell supernatant after 16 hours of stimulation. (C) Theoretical view of a proposed PYRIN inflammasome dependent IL-1 receptor antagonist altering mechanism underlying FMF pathogenesis. Significance of LPS and LPS/C3 activation was tested by repeated measures 2-Way Anova, followed by Holm-Sidak comparison of the main LPS- and LPS/C3 effects (not shown), and the FMF effect for each treatment respectively. ns = non significant, * p<0.05, ** p<0.01, *** p<0.001.
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