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Keywords: arthritis, cryopyrin, pyrin, synovium,

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; CARD, caspase recruitment domain; DIG, digoxygenin; FLS, fibroblast-like synoviocytes; MMP, matrix metalloproteinase; NOD, nucleotide-binding oligomerization domain; OA, osteoarthritis; RA, rheumatoid arthritis; REU, relative expression units.

Summary

Objectives: Rheumatoid arthritis (RA) synovium is characterized by enhanced NF-kB activity and proinflammatory cytokines. Cryopyrin (CIAS-1, NALP-3, PYPAF-1) has been shown to regulate NF-κB and caspase-1 activation. Therefore, we studied the expression of cryopyrin, its effector molecule ASC, and its putative antagonist pyrin in RA and osteoarthritis (OA) synovium and the main two cellular constituents of synovial lining, cultured fibroblast-like synoviocytes (FLS) and macrophages.

Methods: FLS and macrophages were cultured in the presence of inflammatory mediators. Real-time PCR was used to quantify message levels in synovial biopsies and cells. In situ hybridization was employed to localize expression of cryopyrin mRNA.

Results: Cryopyrin mRNA was elevated in RA synovium and detected in both lining and sublining regions. FLS from RA and OA tissue expressed low baseline levels of cryopyrin transcripts that were induced by TNF-α. In contrast, *in vitro*-differentiated macrophages expressed relatively high cryopyrin levels that were further induced by TNF-α, but not by IL-1ß. ASC mRNA levels were comparable in RA and OA tissue, FLS, and macrophages, and were depressed by TNF-α in macrophages. Pyrin expression was higher in RA synovium than in OA tissue, and virtually undetectable in FLS but high in macrophages where it was unchanged by TNF-α treatment.

Conclusion: These results suggest that enhanced cryopyrin levels in RA synovium are due to higher numbers of tissue macrophages, and demonstrate for the first time transcriptional regulation of cryopyrin in a chronic inflammatory disease.

Introduction

Rheumatoid arthritis (RA) is a common inflammatory disease, yet its pathogenesis remains incompletely understood. Synovial tissue from RA patients contains elevated levels of cytokines such as TNF-α and IL-1ß and metalloproteinases such as matrix metalloproteinase (MMP)-1 and MMP-3. The transcription factor NF-κB, which regulates cytokine and MMP expression, is highly activated in RA synovium[1-5]. Hence, pathways that influence NF-κB activation are of interest as potential therapeutic targets for RA.

A family of inflammatory proteins, characterized by the presence of an nucleotide-binding oligomerization domain (NOD), was recently identified[6]. Among them, cryopyrin (PYPAF-1, NALP-3) is a novel protein, encoded by th gene *CIAS1*, with putative inflammatory function[7]. Mutations in *CIAS1* are present in a family of autoinflammatory diseases[7-11]. Common features of these disorders are atypical urticarial rash, as well as rheumatic manifestations (arthralgia, and osseus overgrowth in severe forms). Cryopyrin exerts its function through ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain [CARD]), which functions as an adaptor to downstream pathways[12,13]. When cryopyrin and ASC are co-expressed, they activate caspase-1[14,15], which cleaves pro-IL-1ß to IL-1ß, and upregulate NF-κB activity[12,16,17]. Similar results are observed when ASC is co-expressed with other NOD proteins[18-20]. Pyrin, the protein mutated in familial Mediterranean fever[21] may inhibit cryopyrin-ASC interactions[13].

Cryopyrin and pyrin mRNA is mainly expressed in leukocytes[7,12,22]. ASC is more widely distributed[23]. However, expression patterns of cryopyrin in human inflammatory diseases have not been studied. Because RA synovium is characterized by NF-κB activation and elevated levels of cytokines, the expression of cryopyrin and related proteins were studied in RA and OA synovial tissues as well as in synovial cell subtypes.

Materials and Methods

Patients and tissue preparation

Hip or knee synovial tissue was collected at the time of arthroplasty from RA or OA patients after obtaining informed consent and University of California – San Diego Institutional Review Board approval. Tissues included 12 knees and 2 hips from OA patients; and 8 knees, 4 hips, 1 shoulder and 1 MCP from RA patients. Synovial fragments (size 1-2 mm^2) were excised on ice and immediately frozen in RNAStat-60 reagent (Tel-Test, Friendswood, TX). At least six synovial fragments from each joint were analyzed together to reduce sampling error[24], and only one joint per patient was analyzed. Lining and sublining portions were microdissected from 7 RA and 7 OA cryosectioned synovial tissues as previously described[25].

Cell isolation and culture

FLS were prepared and cultured as previously described[26] and used at passages 3-6. Before experiments, FLS were cultured under serum-reduced conditions (1% FCS) for 2-3 days. Macrophage-like cells were generated from healthy donor PBMC using a

modification of previously reported methods[27]. Briefly, 5 million PBMC/well were allowed to adhere in 24-well plates at 37°C for 3 hours. Non-adherent cells were aspirated, and remaining monocytes were cultured in RPMI containing antibiotics, 10% FCS and 50 ng/ml of M-CSF (R&D Systems, Minneapolis, MN) for 5 days to encourage maturation towards a macrophage-like phenotype. FLS and macrophages were activated using TNF-α (R&D Systems) at 50 ng/ml, and macrophages by LPS (10 ng/ml; E. Coli 0111:B4, Chemicon, Temecula, CA) or IL-1ß (2 ng/ml, R&D Systems). Incubations were terminated by lysis with RNAStat-60.

Real-time quantitative PCR analysis

Synovial and cell cDNA were prepared as in[24] and subjected to real-time quantitative PCR (qPCR) to quantify cryopyrin and ASC transcripts using SYBR Green Universal Master Mix (Applied Biosystems). Primers were as follows: Cryopyrin: forward, 5'-AAAGAGATGAGCCGAAGTGGG, used at 400 nM, reverse, 5'-TCAATGCTGTCTTCCTGGCA, used at 50 nM, product 79 bp; ASC: forward, 5'-GCGAGGGTCACAAACCTT G, reverse, 5'-CTGCTCATCCGTCAGGACCT, both used at 200 nM, product 66 bp. Specificity of qPCR was confirmed by restriction fragment length analysis, melting point and molecular weight of amplicons. MMP-1 qPCR was previously described[24]. Pyrin, IL-1ß, MMP-9, and GAPDH were quantified by TaqMan PCR using pre-developed reagents (Applied Biosystems). Resulting threshold cycle (C_t) data were normalized to standard curves constructed from ConA-stimulated PBMC cDNA[24], yielding cell equivalents. The ratio between the specific cytokine and GAPDH cell equivalents (relative expression units, REU) is reported.

Preparation and transfection of CIAS1-GFP fusion construct

A *CIAS1*- green fluorescent protein (GFP) fusion protein construct was amplified from full length *CIAS1* cDNA[7] in a TOPO cloning vector, and ligated into pEGFP-N1 (Clontech, Palo Alto, CA). CHO cells were transiently transfected with 0.2 µg of CIAS1-GFP DNA using Fugene6 Transfection reagent (Roche Applied Science, Indianapolis, IN) added to 10^5 cells grown in 4-chamber culture slides for 24 hours.

Riboprobe synthesis

A 193 bp fragment (position 257 to 449) was generated by PCR from full-length *CIAS1* and subcloned into pBluescript II KS(-) (Stratagene, La Jolla, CA). This fragment was chosen because of limited homology with other related proteins. The MMP-3 plasmid was described previously[28]. Antisense and sense RNA probes were generated using T7 and T3 (cryopyrin) or T3 and SP6 (MMP-3) RNA polymerases and digoxygenin(DIG)-labelled UTP (Roche). MMP-3 probes were briefly alkaline-hydrolyzed. The resulting riboprobe amount was estimated by dot blot (anti-DIG-AP, 1:5000 dilution, Roche) followed by BCIP/NBT substrate (Vector Laboratories, Burlingame, CA).

In situ hybridization

Cryopyrin and MMP-3 mRNA were visualized in RA synovial paraformaldehyde-fixed cryosections by in situ hybridization. The blocking/acetylation, prehybridization and hybridization procedures were previously described[28] except that 30 ng of DIG-labelled riboprobe was used per section. Slides were washed with three changes of 2xSSC +

50% formamide at 50°C, 50 min total, blocked using 1% sheep serum in Tris-buffered saline (TBS), and incubated overnight with anti-DIG-AP at 1:200 in blocking solution, developed using BCIP/NBT substrate, and counterstained with methyl green.

Data analysis

All qPCR results are shown as mean±SEM of GAPDH-normalized REU. Tissue expression data were log-transformed to acquire normal distributions as detected by Shapiro-Wilk's W test for goodness of fit. Differences in gene expression were detected by Student's t-test, or ANOVA followed by Dunnett's posthoc test when appropriate. Time series data were analyzed using a repeated-measures design. A $p < 0.05$ was considered significant.

Results

Enhanced expression of cryopyrin and pyrin in RA synovium

The expression of cryopyrin, its effector protein (ASC) and its putative antagonist (pyrin) was examined in RA and OA synovium. All three gene products were readily measurable by qPCR in all tissues with the exception of one OA tissue which lacked detectable cryopyrin mRNA. Cryopyrin and pyrin expression was significantly higher in RA synovium($p< 0.030$, and $p<0.0025$ respectively), whereas ASC levels were similar in RA and OA tissues ($p<0.24$) (Fig. 1). Most synovia were from knees, but when only knee tissue data were included in the analysis ($n = 8$ and 12 for RA and OA, respectively) the RA to OA expression ratios were not markedly different from those obtained with the whole data set (ratio = 3.14, 1.89 and 3.22 for cryopyrin, ASC and pyrin, respectively).

Expression of cryopyrin in RA synovial lining and sublining

To determine whether cryopyrin is preferentially expressed in synovial lining or sublining, regions of interest were microdissected from frozen synovial sections and subjected to qPCR. In OA tissues, cryopyrin message was undetectable or barely detectable in whole tissue sections; hence, analysis of microdissected regions was not attempted. In contrast, all RA synovial sections as well as most lining and sublining portions contained detectable levels of cryopyrin mRNA. Interestingly, there was no difference in cryopyrin expression between lining and sublining regions (Table I). Similarly, ASC and pyrin was present at similar levels in RA synovial lining and sublining (Table I).

These findings were confirmed by in situ hybridization in RA synovium. Antisense cryopyrin probe specificity was demonstrated by its hybridization to CHO cells transfected with a cryopyrin-GFP construct (Fig. 2a) at a percentage similar to that observed for GFP protein expression by fluorescence microscopy (not shown). The antisense probe did not hybridize to cells transfected with GFP only, and the sense probe hybridized to neither. MMP-3 expression in synovial sections was used as a control for mRNA quality, and was clearly localized to the synovial lining as expected (Fig. 2b). On the other hand, cryopyrin antisense probe bound to cells in both lining and sublining regions (Fig. 2c). No signal was obtained with cryopyrin sense probe (Fig. 2d).

Taken together, these results show that cryopyrin is expressed in both lining and sublining cells of RA synovium.

Expression of cryopyrin and related proteins in FLS is induced by TNF-α

Based on expression of cryopyrin in the intimal lining, its regulation was evaluated in cultured FLS by qPCR. Cryopyrin message was detectable at low baseline levels, similar in RA and OA FLS lines (Fig. 3a) and independent of whether cells were cultured in 10% or 1% FCS (data not shown). TNF-α transiently induced cryopyrin in both RA and OA FLS (Fig. 3a). There was no difference in cryopyrin expression between RA and OA FLS. To ensure that the FLS remained fully activated throughout the study, MMP-1 mRNA levels were assessed and were shown to be persistently elevated by TNF-α (Fig. 3d).

At baseline, ASC RNA transcripts were present in both RA and OA FLS at levels comparable to those in synovial tissue (Fig. 3b). Intriguingly, TNF-α, significantly increased ASC mRNA levels in RA FLS but not in OA cells (Fig. 3b). Pyrin message was low or undetectable in all baseline FLS cultures, but was induced by TNF-α to the same extent in both RA and OA FLS (Fig. 3c), although due to high variability the increase was not statistically significant. Hence, cryopyrin mRNA is present in very low baseline levels in FLS, but is inducible with TNF-α. On the other hand, ASC message is readily detectable at baseline and increases only in RA FLS following incubation with TNF-α.

Altered expression of cryopyrin and related proteins in macrophages by TNF-α

Recently published data indicated that primary human monocytes increase cryopyrin message in response to LPS and TNF-α[29]. In order to extend these findings to macrophage-like cells, adherence-enriched monocytes were differentiated with M-CSF and 10% FCS for five days and analysed by qPCR. Baseline cryopyrin levels were similar to those observed in synovial tissue, and were increased by LPS or TNF-α, whereas IL-1ß had no effect (Fig. 4a). As a control for activation, IL-1ß message was significantly induced by both LPS and TNF-α. IL-1ß message did not increase significantly after stimulation with IL-1ß cytokine. However, MMP-9 (another macrophage activation marker) mRNA significantly increased following IL-1ß stimulation (REU = 15.3±3.6 and 26.8±2.2 for control and IL-1ß stimulated, respectively, $p = 0.05$), indicating that macrophage activation was achieved with all three stimuli. ASC message levels, which were similar to those in synovial tissue at baseline, declined upon stimulation with LPS or TNF-α whereas IL-1ß again had no effect (Fig. 4b). Finally, pyrin mRNA remained unchanged after activation with either cytokine, whereas LPS stimulated its synthesis (Fig. 4b).

Macrophages were then stimulated with TNF-α for 1 to 24 h, and gene expression was analyzed. Interestingly, expression of cryopyrin increased rapidly, then declined, then rose again in a biphasic response (Fig. 5a). In the same experiment, IL-1ß message remained upregulated for the entire incubation time with TNF-α (Fig. 5d), suggesting that the macrophages were in a continuous state of activation. The earlier observed drop in ASC expression became statistically significant at 8 and 18 hours (Fig. 5b).

However, pyrin mRNA levels remained unchanged throughout the experiment (Fig. 5c). These results demonstrate that cryopyrin message is inducible by TNF-α but not by IL-1ß in macrophages, whereas expression of its effector ASC is lowered and its putative antagonist pyrin remains unchanged.

Discussion

Cryopyrin is a recently identified member of a family of proteins (designated as NOD, NALP, or CATERPILLAR) which modulates pathways crucial to inflammation and apoptosis, such as NF-κB and caspase activation[6]. This protein family is characterized by the presence of a PYRIN or CARD domain, a central NOD domain, and C-terminal leucin-rich repeats (LRR)[6]. Proteins containing a CARD can interact directly with caspases, whereas PYRIN domain-containing proteins such as cryopyrin require the adaptor protein ASC, which contains a PYRIN linked to a CARD, as an intermediary.

In the current study, expression of cryopyrin and related proteins in late-stage rheumatoid synovitis were characterized. Of interest, cryopyrin levels were enhanced in RA synovium over those in OA tissue. OA synovia was used for comparison because of its comparable cellularity to RA as well as its availability. These results suggest either that the resident cells contain elevated cryopyrin message levels, or alternatively that an influx of cells preferentially expressing this gene occurs in RA synovium. In order to elucidate this issue, regional differences in cryopyrin expression were first examined by qPCR on microdissected fragments as well as by in situ hybridization. By both methods, cryopyrin expression was observed in synovial lining as well as sublining, whereas the control mRNA species, MMP-3, was observed mostly in lining by in situ hybridization as earlier described[28]. The cells expressing cryopyrin in the intimal lining could be macrophages and/or FLS (i.e. Type I and/or Type II synoviocytes). Infiltrating macrophages, resident fibroblasts, or other mononuclear cells could contribute to sublining expression.

In tissue distribution studies using Northern blot, significant cryopyrin expression was restricted to leukocytes[7]. By qPCR, cryopyrin message was most abundant in monocytes[12]. In the present studies, cryopyrin mRNA was found in both FLS and in monocyte-derived macrophages. However, macrophages contained substantially more cryopyrin message per cell than FLS (compare Fig. 3 with Figs. 4 and 5), at levels comparable to those in synovial tissue. Hence our results are most consistent with the notion that the high cryopyrin levels in RA tissue are due to increased levels of tissue macrophages.

The rheumatoid synovium is characterized by high levels of cytokines, mostly of macrophage and fibroblast origin[30]. There has been significant discussion regarding the evolution and transformation of the rheumatoid synovium during the course of disease. We evaluated synovia from established disease at arthroplasty, wherein a small cohort could be better defined with respect to disease state in the sampled joint and patients' current treatment regimen. Among the cytokines, TNF-α contributes significantly to disease progression as demonstrated by the clinical success of TNF inhibitors in the treatment of rheumatoid arthritis. A recent study[29] demonstrated that TNF-α rapidly

induces cryopyrin message in human monocytes. In the current study, we extended these findings to differentiated macrophages and also showed that IL-1ß, another prevalent cytokine in RA, does not upregulate cryopyrin. This might indicate a requirement for TNF-α signal transduction pathways, although LPS (which also induced cryopyrin mRNA) signals at least partly through MyD88, a IL-1ß signaling molecule, suggesting that the LPS response might be due to activation of alternative pathways. Surprisingly, TNF-α also increased cryopyrin mRNA levels in FLS. To our knowledge this is the first time that a cell type other than leukocytes have been shown to regulate a NOD protein in response to a cytokine. The difference in expression patterns in the two cell types is intriguing: in FLS, expression is transient, whereas in macrophages two phases of expression are observed. It is currently unclear how these results of relatively short-term expression in both cell types relate to cryopyrin levels in a condition characterized by ongoing cytokine secretion such as RA.

The adaptor protein of cryopyrin, ASC, was previously identified in many cell types and organs[23]. In our hands, ASC expression in FLS and macrophages was similar, and comparable to that in synovial tissue. Thus, both FLS and macrophages likely contribute to ASC expression in synovium. In response to TNF-α, an intriguing difference between RA and OA FLS was found in that RA cells upregulated ASC message while OA FLS did not, whereas both cell types produced high levels of MMP-1 indicating full activation. Even more unexpectedly, macrophages <u>down</u>regulated ASC message levels 50-70% in response to TNF-α while increasing their levels of mRNA coding for IL-1ß (and cryopyrin). This appears to indicate differential regulation of ASC by inflammatory mediators in FLS and macrophages.

The protein pyrin contains a PYRIN domain, linked to C-terminal sequence unique among PYRIN domain proteins[31]. In earlier studies, High pyrin mRNA levels were observed in neutrophils and monocytes[22], and also identified in peritoneal and skin fibroblasts[32] as well as in FLS[33] where it was inducible by TNF-α. In the present set of studies, pyrin expression in RA synovium was elevated about three-fold over the level in OA synovial tissue. Baseline levels in FLS were virtually undetectable, and TNF-α appeared to induce expression to a variable extent. In contrast, macrophages contained readily detectable pyrin message that remained constant in the presence of TNF-α (although it was induced by LPS). Comparing tissue, macrophage and FLS expression levels of pyrin (Figs. 1, 3, and 5) suggest that FLS contribute only a minor portion of pyrin mRNA in RA synovium. Hence, as for cryopyrin, the data are consistent with the notion that the elevated pyrin expression in RA synovium are due to higher macrophage numbers.

Several groups have studied interaction among cryopyrin, pyrin, and ASC in plasmid overexpression systems. Pyrin was shown to compete with caspase-1 for binding to ASC thereby exerting a modulating effect on IL-1ß production[34]. Pyrin has also been proposed as an antagonist of cryopyrin-ASC interactions[13]. Thus, pyrin might interfere with cryopyrin-mediated cytokine production at several points in the signaling cascade. However, when cryopyrin was overexpressed in the absence of ASC, it reduced TNF-α-stimulated NF-κB translocation[29] as well as IL-1ß secretion[14]. The physiological

relevance of these findings is unclear because both cryopyrin and ASC are present in cells of myeloid lineage. In fact, when ASC was added to cryopyrin, IL-1ß secretion was instead enhanced in a cryopyrin plasmid-dose response manner[14].

These data suggest that the outcome of interactions among cryopyrin, ASC and pyrin depend on their relative balance. Hence, our current observations of differential regulation of cryopyrin and ASC mRNA levels in macrophages may have significance. Whereas the early cryopyrin peak occurs while ASC levels remain steady, the later phase of cryopyrin expression coincides with a significant drop in ASC mRNA. It is tempting to speculate that the lowered ASC levels allow cryopyrin to exert its inhibitory function, although further studies are needed to understand the precise nature of such interactions. The identification of several cryopyrin-like proteins within the human genome[35] suggest that other factors may influence the function of cryopyrin and ASC.

In conclusion, cryopyrin and pyrin mRNA were elevated in RA synovium when compared to OA synovium. Cryopyrin was equally expressed in synovial lining and sublining and identified within cells in both areas by in situ hybridization. Both FLS and macrophages in vitro expressed cryopyrin, but macrophages contained much higher levels both at baseline and following TNF-α induction. mRNA levels for the adaptor, ASC, were similar among RA and OA synovium, FLS, and macrophages. On the other hand, the putative cryopyrin antagonist, pyrin was virtually undetectable in FLS but expressed in macrophages. These data demonstrate for the first time cryopyrin regulation in a chronic inflammatory condition and set the stage for future studies of the functional significance of cryopyrin, ASC and pyrin in RA.

Acknowledgements
The authors wish to thank Suzanne Beal for help with preparation of in situ hybridization slides, and Justin Anderson and James Mueller for assistance with cloning, transfections, and monocyte isolation.

Table I. Expression of cryopyrin and ASC in RA synovial lining and sublining.

Gene product	RA synovial lining (REU) [a]	RA synovial sublining (REU)	p
Cryopyrin	2.50 ± 0.74	4.44 ± 1.70	0.56
ASC	3.97 ± 0.60	4.28 ± 0.73	0.99
Pyrin	0.68 ± 0.23	0.83 ± 0.27	0.70

[a] Data are expressed as mean and SEM of GAPDH-normalized REU from 2 microdissected regions each of 7 (cryopyrin and ASC) or 4 (pyrin) RA synovial cryosections. There was no significant difference in gene expression between lining and sublining regions for any transcript.

Figure legends

Fig. 1. Expression of cryopyrin, ASC and pyrin mRNA in synovial tissue from RA and OA patients undergoing joint replacement surgery, as determined by real-time quantitative PCR. REU (relative expression units) data are standardized to known dilutions of PBMC cDNA and normalized by GAPDH to control for cellularity. Results are from an n of 14 patients per group. Asterisk indicates $p < 0.05$ by Student's t-test on log-transformed data. Both cryopyrin and pyrin message was significantly elevated in RA.

Fig. 2. Detection of cryopyrin mRNA in synovial tissue by in situ hybridization. A, CHO cells transfected with a cryopyrin-GFP fusion construct and hybridized with cryopyrin antisense probe to demonstrate its specificity. No signal was obtained with cryopyrin sense probe (not shown). B, RA synovium hybridized with MMP-3 antisense probe shows typical lining distribution. MMP-3 sense probe did not hybridize (not shown). C, Cryopyrin mRNA is expressed in both lining and sublining in RA synovium as shown by antisense probe. D, cryopyrin sense probe did not hybridize to RA synovium (serial section, same area as in C).

Fig. 3. Expression of cryopyrin (a), ASC (b) and pyrin (c) mRNA in FLS after stimulation with TNF-α (50 ng/ml) at time zero. Results are from real-time quantitative PCR studies of 3 cell lines each and are expressed as GAPDH-normalized REU as in Fig. 1 legend. Asterisk and ampersand indicates $p < 0.05$ from time zero and between RA and OA, respectively, by repeated-measures ANOVA. Expression of cryopyrin was transiently elevated in both RA and OA FLS (a). ASC was upregulated in RA but not OA FLS (b).Pyrin was not detectable at baseline but was induced (c). As a control, continuous MMP-1 expression demonstrates that FLS were fully activated (d).

Fig. 4. Expression of cryopyrin (a), ASC and pyrin (b) mRNA in macrophage-like cells differentiated from healthy donor monocytes by 5 day culture in M-CSF and serum. Cells were treated with LPS (10 ng/ml), TNF-α (50 ng/ml), or IL-1ß (2 ng/ml) for 18 h before real-time quantitative PCR analysis. Results are from three donors and expressed as GAPDH-normalized REU as in legend to Fig. 1. Asterisk indicates $p < 0.05$ to control, untreated cells by single ANOVA and Dunnett's post-hoc test. Cryopyrin message was induced by LPS and TNF-α, whereas ASC message levels decreased under the same conditions. Pyrin was only elevated by LPS but not by either cytokine. IL-1ß message was significantly upregulated by LPS and TNF-α (a), whereas IL-1ß itself upregulated MMP-9 expression (see text) indicating macrophage activation in all cases.

Fig. 5. Time course of mRNA expression in monocyte-derived macrophage-like cells in response to treatment with TNF-α (50 ng/ml). Results are from three donors and expressed as GAPDH-normalized REU as in legend to Fig. 1. Asterisk indicates $p < 0.05$ from time zero by repeated-measures ANOVA. Cryopyrin message was induced in two phases (a), whereas ASC levels fell (b). Pyrin remained unaltered (c). Cells were fully activated as indicated by continuous expression of IL-1ß (d).

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Fig. 1.

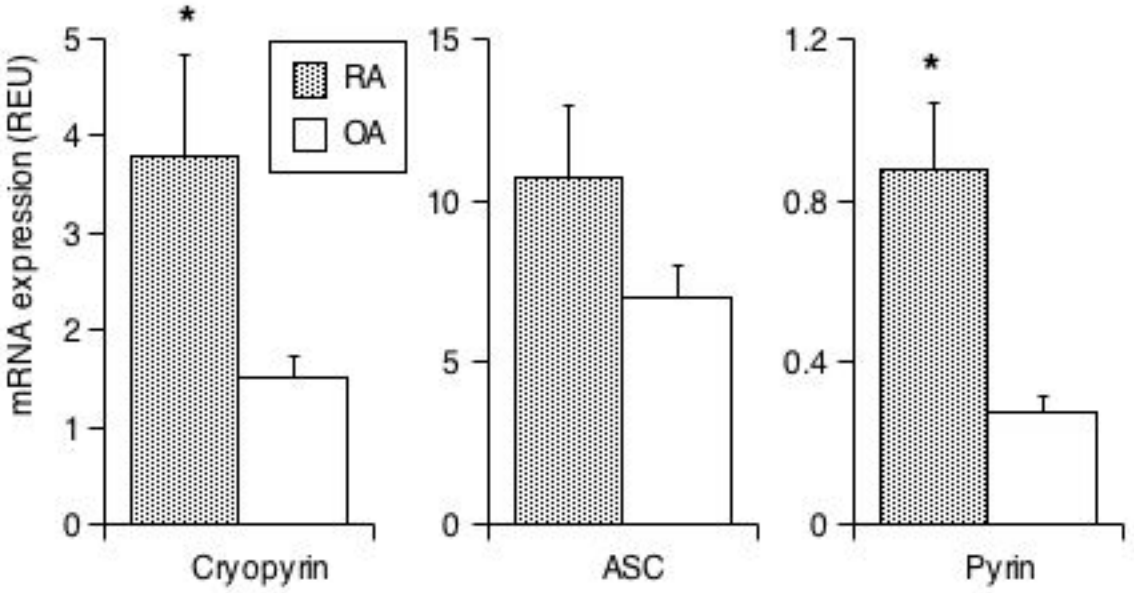

mRNA expression (REU)
RA
OA
*
*
5
4
3
2
1
0
15
10
5
0
1.2
0.8
0.4
0
Cryopyrin
ASC
Pyrin

Fig. 2.

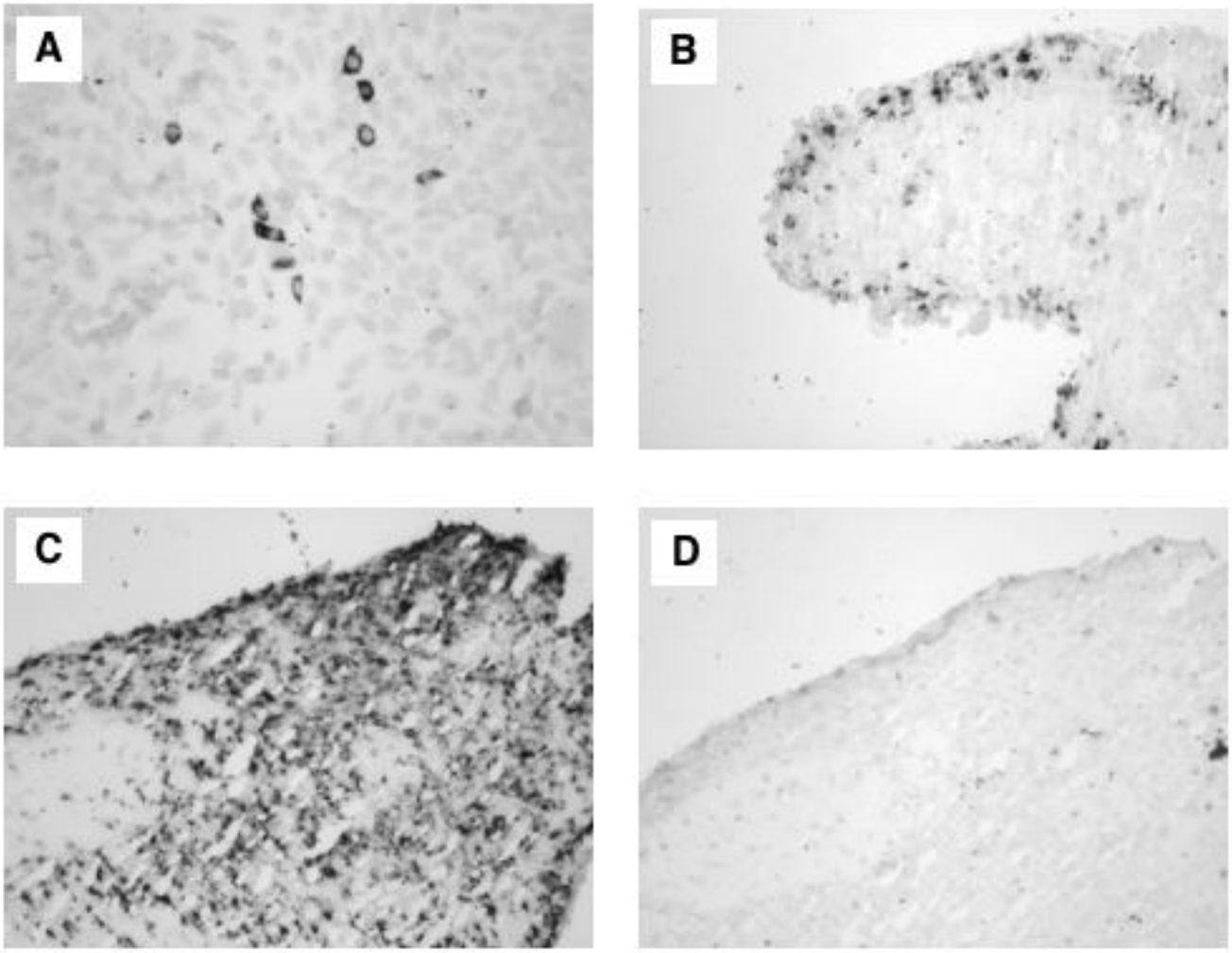


Fig. 3.

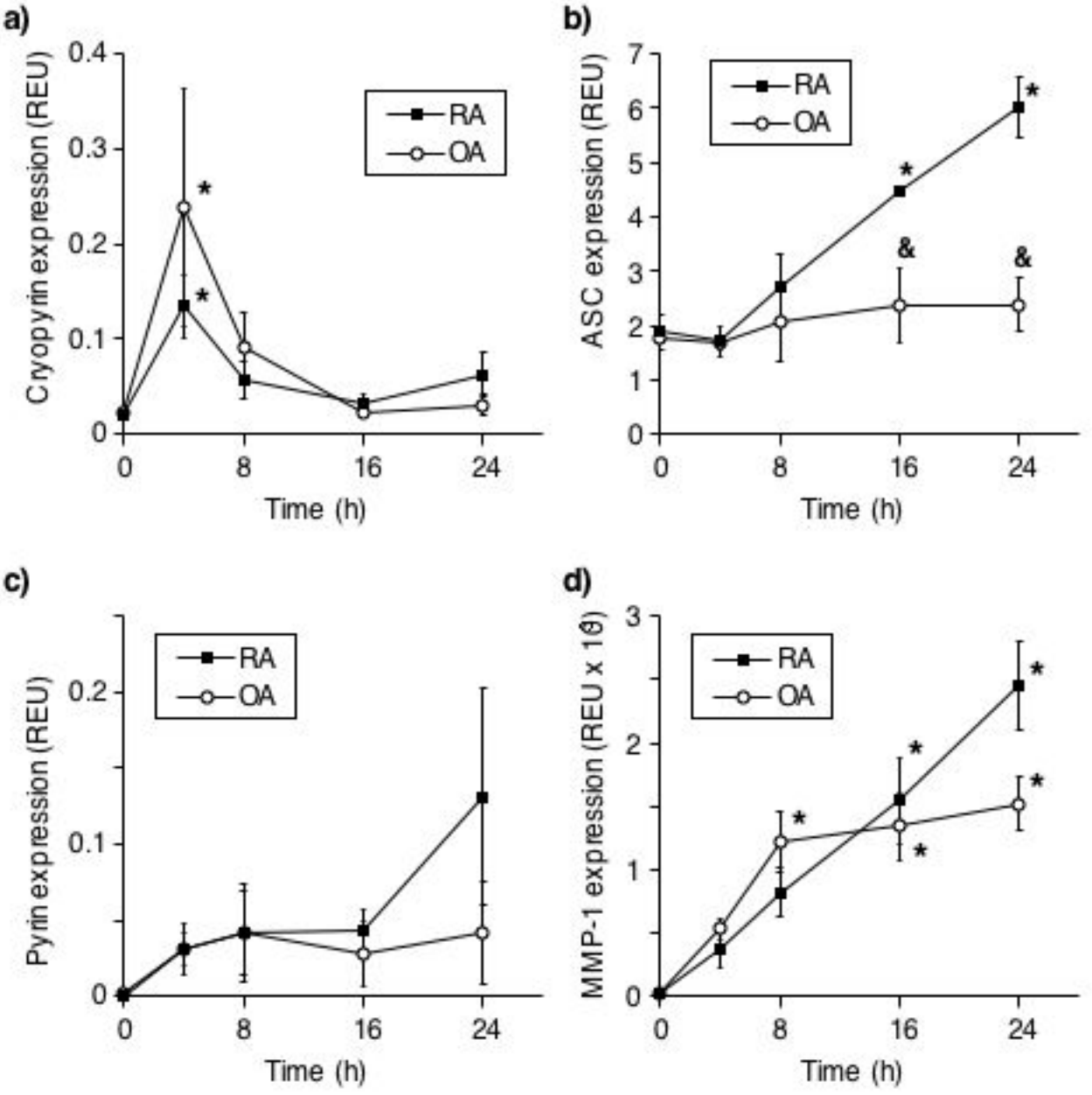

a)
Cryopyrin expression (REU)
0.4
0.3
0.2
0.1
0
RA
OA
*
*
0
8
16
24
Time (h)
b)
ASC expression (REU)
7
6
5
4
3
2
1
0
RA
OA
*
*
&
&
0
8
16
24
Time (h)
c)
Pyrin expression (REU)
0.2
0.1
0
RA
OA
0
8
16
24
Time (h)
d)
MMP-1 expression (REU x 10)
3
2
1
0
RA
OA
*
*
*
*
0
8
16
24
Time (h)

Fig. 4.

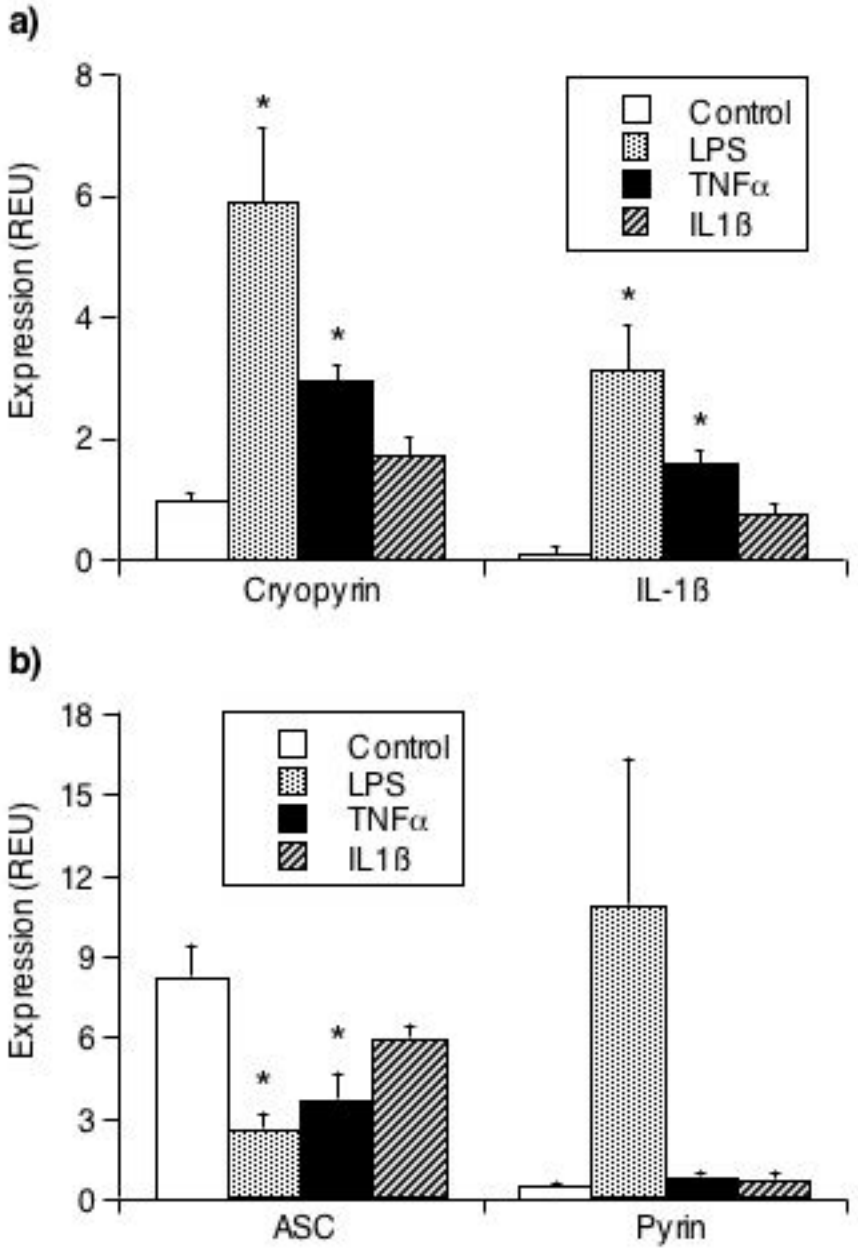
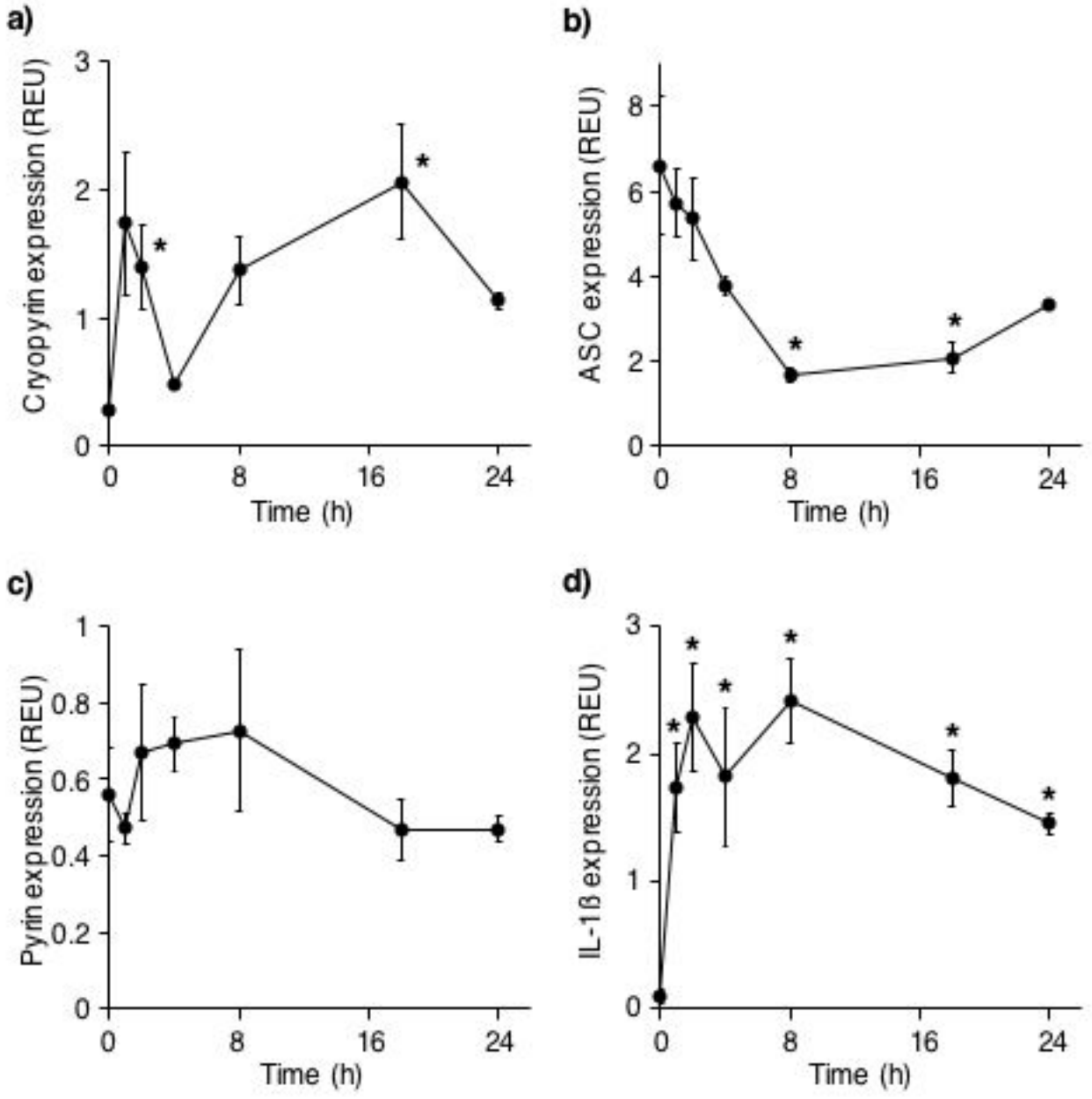

a)
Expression (REU)
8
6
4
2
0
Control
LPS
TNFα
IL1ß
*
Cryopyrin
IL-1ß
b)
Expression (REU)
18
15
12
9
6
3
0
Control
LPS
TNFα
IL1ß
ASC
Pyrin

Fig. 5.


a)
Cryopyrin expression (REU)
Time (h)
b)
ASC expression (REU)
Time (h)
c)
Pyrin expression (REU)
Time (h)
d)
IL-1ß expression (REU)
Time (h)