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J Immunol published online 4 February 2019

<http://www.jimmunol.org/content/early/2019/02/01/jimmunol.1701531>

Supplementary Material <http://www.jimmunol.org/content/suppl/2019/02/01/jimmunol.1701531.DCSupplemental>

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



The Long Pentraxin 3 Contributes to Joint Inflammation in Gout by Facilitating the Phagocytosis of Monosodium Urate Crystals

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The purpose of this study was to investigate the role of pentraxin 3 (PTX3), a pivotal component of the innate immune system, in gout. Levels of PTX3 and IL-1 β in human samples were evaluated by ELISA. Development of murine gout was evaluated through the levels of cytokines (PTX3, CXCL1, and IL-1 β) and neutrophil recruitment into the joint cavity. Phagocytosis of monosodium urate (MSU) crystals and caspase-1 activation were determined by flow cytometer. Acute gout patients showed elevated concentration of PTX3 in plasma and synovial fluid as compared with healthy and osteoarthritic subjects. Moreover, there was a positive correlation between intra-articular PTX3 and IL-1 β levels. PTX3 was induced in the periarticular tissue of mice postinjection of MSU crystals. Importantly, Ptx3-deficient mice showed reduced inflammation in response to MSU crystal injection compared with wild-type mice, including reduction of neutrophil recruitment into the joint cavity and IL-1 β and CXCL1 production. Interestingly, addition of PTX3 in vitro enhanced MSU crystal phagocytosis by monocytes and resulted in higher production of IL-1 β by macrophages. This contribution of PTX3 to the phagocytosis of MSU crystals and consequent production of IL-1 β occurred through a mechanism mainly dependent on Fc γ RIII. Thus, our results suggest that PTX3 acts as a humoral pattern recognition molecule in gout facilitating MSU crystal phagocytosis and contributing to the pathogenesis of gouty arthritis. *The Journal of Immunology*, 2019, 202: 000–000.

Gout is the most common form of inflammatory arthritis worldwide among men and postmenopausal women. Its incidence and prevalence have increased mainly because of lifestyle changes, such as a higher alcohol and purine-rich diet consumption, and it is closely associated to the metabolic syndrome (1, 2). Joint inflammation in gout occurs because of the deposition of monosodium urate (MSU) crystals predominantly in peripheral joints and surrounding tissues and mainly in individuals with chronic hyperuricemia. Acute gout attacks, although characterized by a self-limited inflammation, are extremely painful and can lead to joint disability. Prolonged deposition of MSU crystals can result in irreversible joint damage with bone erosion and development of s.c. tophi (3).

The initial events of MSU crystal-triggered inflammation occur after the contact and phagocytosis of the crystals by synovial

phagocytes. The internalization of MSU crystals results in the assembly of NACHT, LRR, and PYD domains-containing protein 3 (NLRP3)/apoptosis-associated speck-like protein containing a CARD (ASC)/caspase-1 inflammasome that culminates in the release of the mature form of IL-1 β (4), a cytokine with a crucial role in gouty arthritis. IL-1 β promotes the production of different chemoattractants that cause early neutrophil swarm to the joint and lead to joint inflammation, damage, and pain. However, the mechanisms underlying the recognition of MSU crystals by phagocytes have not yet been completely elucidated. Some studies have demonstrated that MSU crystals bind to plasma proteins, such as complement components IgG and IgM (5–7). The opsonisation of MSU crystals by these molecules enables direct contact with their receptors on the leukocyte surface, as demonstrated in neutrophils through CR3 (CD11b/CD18) and Fc γ RIIIB (CD16), which

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Received for publication November 7, 2017. Accepted for publication January 4, 2019.

This work was supported by contributions from the Brazilian National Council for Scientific and Technological Development, the National Institute of Science and Technology in Dengue and Host-Parasite Interactions (465425/2014-3), the Minas Gerais State Agency for Research and Development (FAPEMIG), the São Paulo Research Foundation under Grant Agreement 2013/08216-2 (Center for Research

in Inflammatory Diseases), the University of São Paulo Núcleo de Pesquisa em Doenças Inflamatórias under Grant Agreement 11.1.21625.01.0, and the European Commission (ERC – 669415 to A.M.), the Italian Association for Cancer Research (AIRC, IG, and 5x1000), Fondazione Cariplo (2015-0564), the Italian Ministry of Health (RF-2013-02355470), and the Italian Ministry of Education, University and Research (PRIN 2015YYKPN).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CytD, cytochalasin D; MSU, monosodium urate; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; OA, osteoarthritic; PTX3, pentraxin 3; RA, rheumatoid arthritis; rPTX3, recombinant PTX3; WT, wild-type.

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bind crystal-bound iC3b and IgG, respectively (8). However, the receptors involved in the phagocytosis of MSU crystals by mononuclear phagocytes need to be demonstrated.

The soluble pattern recognition molecules, including complement system, natural Abs, and pentraxins constitute the humoral arm of the innate immune response (9). Pentraxins are a superfamily of evolutionarily conserved multimeric proteins, which is divided into short (C-reactive protein and serum amyloid P component) and long pentraxins. Pentraxin 3 (PTX3), the prototype of the long pentraxin family, is produced and released by a variety of cell types, including phagocytes, dendritic cells, fibroblasts, and endothelial cells under different stimuli, such as LPS, IL-1, and TNF- α (10–12). PTX3 can interact with a variety of pathogens (e.g., *Aspergillus fumigatus* and *Pseudomonas aeruginosa*) and has opsonic activity facilitating their phagocytosis (13–15) through interaction with Fc γ R, which have been identified as pentraxin receptors (16). Moreover, PTX3 is involved in the pathogenesis of acute and chronic sterile inflammatory diseases, including rheumatoid arthritis (RA) and ischemia/reperfusion (17, 18). However, the involvement of PTX3 in gout has not yet been described.

The present study was designed to investigate the role of PTX3 in gouty inflammation. The analysis of samples obtained from gouty patients demonstrated an increase of PTX3 levels and a positive correlation of this protein with IL-1 β . Using genetically deficient mice, we demonstrate that PTX3 leads to an increase of MSU crystal phagocytosis by mononuclear phagocytes by a mechanism dependent on Fc γ R. This event culminates in increased inflammasome activation and IL-1 β secretion by these cells. Altogether, our results suggest that PTX3 is an important molecule that facilitates MSU crystal-driven inflammation in gouty arthritis.

Materials and Methods

Samples of patients diagnosed with gout

Two Brazilian groups of patients diagnosed with acute gout flares (<48 h after gout attack), according to the 2015 Gout Classification Criteria, were used in this study: one from Rio de Janeiro (A) and other from Ribeirão Preto (B). One Brazilian group (from Ribeirão Preto) of patients diagnosed with osteoarthritis of the knee, according to the Classification of American Rheumatism Association, was also used in this study. Synovial fluid collected from the knee and blood samples were collected in EDTA tubes, centrifuged (2,000 \times g for 10 min), and the supernatant kept at -20°C. A fresh sample of synovial fluids from gout and osteoarthritis underwent MSU crystal identification by compensated polarized light microscopy. All patients provided informed consent to participate in the study, which was approved by Comitê de Ética em Pesquisa under protocol number 1.297.041, and by Certificado de Apresentação para Apreciação Ética (Comissão Nacional de Ética em Pesquisa) under protocol number 50373815.4.0000.5259 in Rio de Janeiro and 4971/2012 in Ribeirão Preto.

Animals

The experiments with mice were performed in two different laboratories: Italy and Brazil. Eight- to twelve-week-old mice were housed in a controlled environment and had free access to commercial chow and filtered water. All mice used were on C57BL/6 genetic background. All efforts were made to minimize the number of animals used and their suffering. Italy: Ptx3-deficient mice were generated as described (13). FcR γ -deficient mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All colonies were housed and bred in the specific pathogen-free animal facility of Humanitas Clinical and Research Center in individually ventilated cages. Wild-type (WT) mice were obtained from Charles River Laboratories, Calco, Italy. Procedures involving animals' handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale, suppl. 40, February 18, 1992; Decreto Legislativo n.26, March 4, 2014) and international law and policies (European Economic Community Council Directive 2010/63/EU, official journal L 276/33, 22-09-2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). The study was approved by the Italian Ministry of Health (approval 71/2012-B).

Brazil: Fc γ RIIB- and Fc γ RIII-deficient mice were generated as previously described. These mice were a kind gift of Dr. J. M. Penninger and were bred in our facilities. WT mice were purchased from the animal facility of Federal University of Minas Gerais, Brazil. The experiments were made according to the ethical principles in animal experimentation of our institution, and the experimental protocol was approved by the Ethics Committee in Animal Experimentation of the University (protocol 35/2016-Comissão de Ética no Uso de Animais/Universidade Federal de Minas Gerais).

Experimental model of acute gout (MSU crystal-induced inflammation)

Mice were placed under anesthesia (80:15 mg/kg ketamine:xylazine, i.p.; Syntec, São Paulo, Brazil) and were injected with MSU crystals (100 μ g/cavity) into the tibiofemoral knee joint. MSU crystals were prepared as previously described (19). Inflammatory parameters were evaluated at 6 or 15 h after injection of MSU crystals. Mice were euthanized in a CO₂ chamber, followed by cervical dislocation, and the articular cavity was washed with PBS containing 3% BSA for leukocyte counts. The number of cells was determined in a Neubauer chamber after staining with Turk solution. Differential counts were performed by Shandon CytoSpin III (Thermo Shandon, Frankfurt, Germany) preparations by evaluating the percentage of each leukocyte on a slide after staining with May-Grunwald-Giemsa (20). Periarticular tissue was removed from the joints for cytokine evaluation by ELISA.

ELISA

Periarticular tissues were collected from joints and homogenized with PBS containing antiproteases (21). Samples were centrifuged, and the supernatant was used to quantify PTX3, IL-1 β , and CXCL1. Plasma and synovial fluid from patients were used for determination of PTX3 and IL-1 β levels. A commercially available ELISA was used in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Phagocytosis of MSU crystal assay

One hundred microliters of mouse whole blood were incubated with MSU crystals (150 μ g/ml) for 15 min at 37°C. Previously, these crystals were incubated or not with recombinant PTX3 (rPTX3) for 1 h. We tested different concentrations of rPTX3 and presented those results that had significance compared with the groups without rPTX3. Human cells were isolated with a density gradient centrifugation in accordance with the manufacturer's instructions (Histopaque 1077 and Histopaque 1119; Sigma-Aldrich, St. Louis, MO). PBMC were plated for 2 h to allow the adherence, and then these cells or isolated neutrophils (5×10^5) were stimulated as murine cells described above. Purity was checked using cytospin preparation, and all samples used exceeded 90% purity. After stimulation with MSU crystals, samples were placed on ice to block phagocytosis, and red cells were lysed by adding cold ammonium chloride lysis solution (pH 7.2). As a control of phagocytosis, cells were preincubated with 20 μ M cytochalasin D (CytD) (Sigma-Aldrich) for 1 h before the stimulation with MSU crystals. Human monocytes were stained for CD14. Murine samples were stained for monocytes (CD45⁺CD11b⁺Ly6G^{high}) and neutrophils (CD45⁺CD11b^{high} Ly6G^{high}) (eBiosciences, BD Pharmingen). Samples were then fixed with 1% paraformaldehyde. The phagocytosis of MSU crystals was determined by analysis of side scatter change in flow cytometry, as previously described (22). The values were represented by relative percentage to the group of cells incubated only with MSU crystals. The analyses were performed with FlowJo software (Tree Star).

Caspase-1 activity assay

For the detection of active caspase-1, FAM FLICA Caspase-1 Assay Kit (ImmunoChemistry) was used. Monocytes isolated from human whole blood (as previously described) were primed with LPS (100 ng/ml) for 1 h and then stimulated with MSU crystals (MSU 150 μ g/ml) for 6 h. Cells were stained with FLICA in accordance with the manufacturer's instructions and also for CD14 for identification of monocytes. The analyses were performed with FlowJo software (Tree Star).

Preparation and culture of murine macrophages

Mice received an i.p. injection of a 3% thioglycolate solution, and macrophages were collected by peritoneal lavage 4 d later. Cells were plated for 2 h, and later, nonadherent cells were removed. Cells were cultured in RPMI medium complemented with 10% FCS, penicillin/streptomycin, and L-glutamine and kept in a humidified incubator at 37°C with 5% CO₂ overnight. Cells were primed for 1 h with LPS

(100 ng/ml) (Sigma-Aldrich) and then stimulated with MSU crystals (150 μ g/ml) for 6 h. The supernatant was used for IL-1 β detection by ELISA.

Statistical analysis

All results are presented as the means \pm SEM. Results were tested for normality, and differences between groups were evaluated using one-way ANOVA followed by Newman–Keuls posttest, Student *t* test, Mann–Whitney *U* test, or Pearson correlation as specified. The level of significance was set at $p < 0.05$. Statistics were calculated with GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA).

Results

PTX3 positively correlates with IL-1 β in patients with acute gout attack

To address the relevance of PTX3 in gout, we first evaluated the concentration of PTX3 in plasma and synovial fluid of patients diagnosed with acute gout. In group A, there was an increase of PTX3 levels in plasma of patients with gout compared with healthy volunteers. Interestingly, the levels of PTX3 were higher in synovial fluid as compared with plasma in those patients (Fig. 1A). In group B, the concentration of PTX3 in synovial fluid was much higher in gout patients compared with osteoarthritic (OA) patients (Fig. 1B). Because IL-1 β is a key proinflammatory marker in gout (23–25), we quantified this cytokine in those samples. In both groups, IL-1 β was detected in synovial fluid of gout patients (data not shown). Importantly, there was a positive correlation between PTX3 and IL-1 β in synovial fluid during acute gout in group A (Fig. 1C) and group B (Fig. 1D). However, there was no positive correlation between PTX3 and IL-1 β in plasma of group A (data not shown). Collectively, the increased level of PTX3 during gout flares and its association with IL-1 β indicate a participation of PTX3 in the early events of gouty inflammation.

PTX3 contributes to gouty inflammation

To investigate whether PTX3 contributed to the inflammatory response observed in gout, we used an experimental model of acute gout in mice through the injection of MSU crystals into the tibiofemoral joint (20). In this model, the peak of inflammation ranges between 6 and 15 h after the insult. Injection of MSU crystals caused an increase of PTX3 levels in inflamed joints 6 h postinjection when compared with saline-injected joints, and this increase was even greater at the 15th hour (Fig. 2A). MSU crystals also caused an increase of IL-1 β , CXCL1, and influx of neutrophils to the joint compared with control group (Fig. 2B–D). Ptx3-deficient mice had a reduction in these inflammatory parameters following the injection of MSU crystals as compared with WT mice (Fig. 2B–D). Of note, the injection of rPTX3 alone was not able to cause an inflammatory response in the joint (data not shown). Altogether, these data strengthen the contribution of PTX3 to MSU crystals–induced inflammation.

PTX3 facilitates the phagocytosis of MSU crystals and cell activation

PTX3 is known to interact with different microorganisms. It has opsonic activity and contributes to pathogen phagocytosis and clearance (13–15). Thus, we investigated whether PTX3 could be involved in the phagocytosis of MSU crystals. To set up an assay of phagocytosis of MSU crystal (Supplemental Fig. 1) and its consequence on IL-1 β release, we first treated murine whole blood with CytD, an inhibitor of actin polymerization that impairs phagocytosis, before MSU crystal stimulation and performed the analysis of phagocytosis of MSU crystals by flow cytometry. CD45⁺CD11b⁺Ly6C^{high} monocytes phagocytosed MSU crystals, and this was significantly reduced after CytD treatment (Fig. 3A).

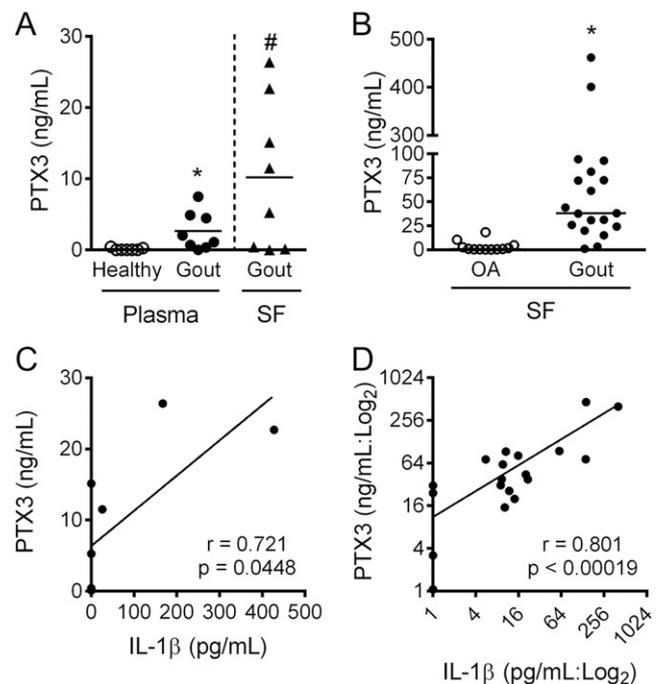


FIGURE 1. The amount of PTX3 is increased in gouty patients and positively correlated with IL-1 β levels. The amount of PTX3 was measured by ELISA in plasma and synovial fluid (SF) of healthy and gouty patients from cohort A (**A**). The amount of PTX3 was measured by ELISA in SF of OA and gouty patients from cohort B (**B**). Pearson correlation analysis between PTX3 and IL-1 β in SF obtained from cohort A (**C**) and B (**D**). The limits of detection of ELISA kit used in this study are as follows: 3.90–250 pg/ml for IL-1 β and 218.75–14,000 pg/ml for PTX3. * $p < 0.05$ compared with its control. # $p < 0.05$ between plasma and SF of gouty patients in (A).

MSU crystals stimulated the release of IL-1 β by LPS-primed peritoneal macrophages. Incubation with CytD showed decreased release of IL-1 β levels in the supernatant, confirming that phagocytosis of MSU crystals is necessary for IL-1 β maturation and release (Fig. 3B). This effect was indeed dependent on the phagocytosis of MSU crystals because CytD did not reduce the release of IL-1 β when cells were incubated with ATP, a well-known NLRP3 inflammasome activator (data not shown). Ptx3-deficient monocytes showed defective phagocytic activity when compared with WT cells. However, the incubation of rPTX3 in whole blood increased the phagocytosis of MSU crystals by monocytes in both WT and Ptx3-deficient cells (Fig. 3C). Peritoneal macrophages incubated with rPTX3 also increased the release of IL-1 β after MSU crystal stimulation in both WT and Ptx3-deficient cells (Fig. 3D). Of note, macrophages stimulated with rPTX3 alone did not produce IL-1 β (data not shown). CD45⁺CD11b^{high}Ly6C^{high} neutrophils from Ptx3-deficient mice had reduced phagocytosis of MSU crystals when compared with WT neutrophils, a phenotype that was rescued by the addition of rPTX3 (Supplemental Fig. 2A).

A similar profile was also observed in human cells. The presence of rPTX3 increased the phagocytosis of MSU crystals by blood CD14⁺ monocytes from healthy donors (Fig. 4A). Also, there was an increase of IL-1 β in the supernatant of cells incubated with rPTX3 and MSU crystals (Fig. 4B). As the cleavage and release of IL-1 β under MSU crystal stimulation is dependent on NLRP3/ASC/caspase-1 (20), we measured the cleavage of caspase-1 in CD14⁺ monocytes. As shown in Fig. 4C, the incubation with rPTX3 increased MSU crystal–dependent caspase-1 activation when compared with cells treated only with MSU crystals. Along

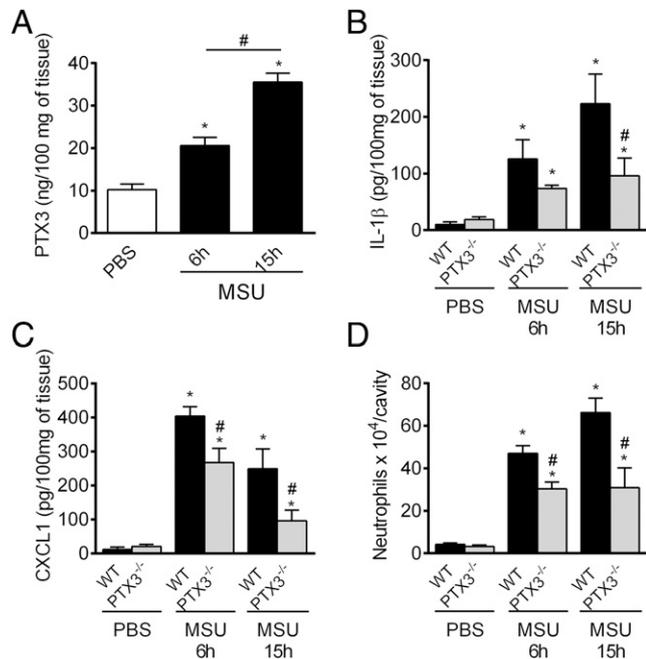


FIGURE 2. PTX3 participates in MSU crystal-induced joint inflammation. WT mice received an intra-articular injection of MSU crystals (100 μ g/cavity), and periarticular tissue was collected at different time points (6 or 15 h after MSU crystal injection) to quantify PTX3 by ELISA (A). Concentrations of IL-1 β (B) and CXCL1 (C) in homogenized periarticular tissue and the accumulation of neutrophils into the joint cavity (D) in WT and Ptx3 $^{-/-}$ mice after 15 h following the injection of MSU crystals. Results are presented as the mean \pm SEM ($n = 5-6$). The experiments were repeated at least twice. ANOVA followed by Newman-Keuls posttest. * $p < 0.05$ compared with PBS-injected mice. # $p < 0.05$ compared with MSU crystal-injected WT mice corresponding to each time point.

the same line, isolated human neutrophils incubated with MSU crystals in the presence of rPTX3 showed greater phagocytosis of the MSU crystals (Supplemental Fig. 2C). Thus, PTX3 facilitates the phagocytosis of MSU crystals and consequently favors the production of IL-1 β in mononuclear phagocytes.

The role of PTX3 in gout depends on Fc γ R s , mainly Fc γ RIII

It has been reported that the interaction of PTX3 with Fc γ R s mediates part of the biological activities of PTX3 (16). Thus, we investigated the contribution of Fc γ R s to PTX3-induced MSU crystal phagocytosis and their role in gouty inflammation. Monocytes from Fc γ R-deficient mice had lower phagocytosis rate in comparison with cells from WT mice. Interestingly, the addition of rPTX3 to MSU crystal-stimulated Fc γ R $^{-/-}$ cells did not increase the phagocytosis of the crystals, in contrast with what we observed with WT cells (Fig. 5A). Next, we tested this response in cells from Fc γ RIIB (an inhibitory Fc γ R)-deficient and Fc γ RIII (a stimulatory Fc γ R)-deficient mice. Monocytes from both Fc γ RIIB $^{-/-}$ and Fc γ RIII $^{-/-}$ mice showed the same levels of phagocytosis of MSU crystals as WT mice. However, cells from WT and Fc γ RIIB $^{-/-}$, but not from Fc γ RIII $^{-/-}$, mice showed increased phagocytosis after the incubation with rPTX3 (Fig. 5B). We found a similar profile when analyzing IL-1 β release (Fig. 5C). WT-, Fc γ R $^{-/-}$ -, Fc γ RIIB $^{-/-}$ -, and Fc γ RIII $^{-/-}$ -primed macrophages stimulated with MSU crystals released more IL-1 β levels in the supernatant (Fig. 5D) than nonstimulated cells. However, the incubation with rPTX3 increased the amount of IL-1 β further only in WT and Fc γ RIIB $^{-/-}$ cells but did not affect IL-1 β release by Fc γ R $^{-/-}$ and Fc γ RIII $^{-/-}$ cells (Fig. 5C, 5D).

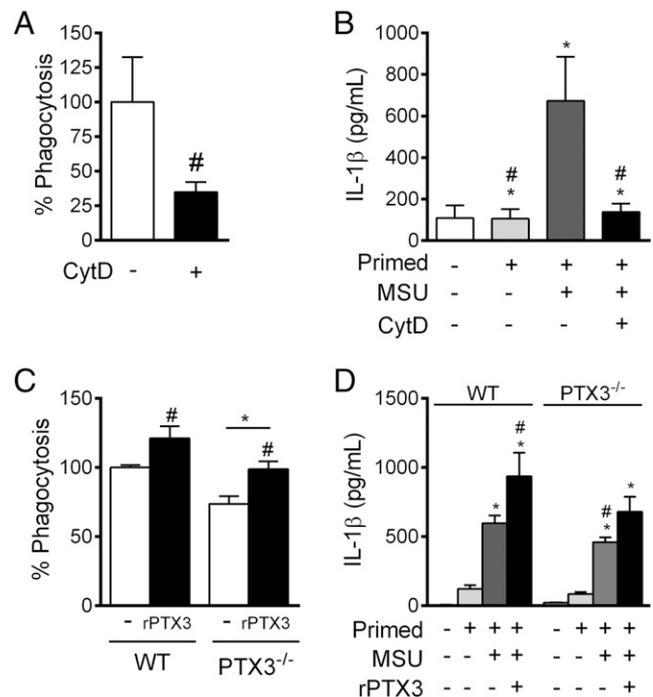


FIGURE 3. PTX3 increases the phagocytosis of MSU crystals and IL-1 β production by mononuclear phagocyte-derived murine cells. The phagocytosis of MSU crystals was evaluated in monocytes CD45 $^+$ CD11b $^+$ Ly6C $^{\text{high}}$ after the incubation of whole blood with the crystals for 15 min and analyzed by side scatter (SSC) change in flow cytometry (A and C). IL-1 β was quantified by ELISA in the supernatant of peritoneal macrophages stimulated with MSU crystals for 6 h (B and D). Cells from WT mice were pretreated with 20 μ M CytD 1 h before the stimulation with MSU crystals (A and B). Cells from WT and Ptx3 $^{-/-}$ mice were stimulated with MSU crystals preincubated or not with rPTX3 (50 μ g/ml) for 6 h (C and D). For priming, peritoneal macrophages were incubated with LPS (100 ng/ml) for 1 h before the incubation with MSU crystals. Results are mean \pm SEM ($n = 5-6$). The experiments were repeated at least twice. Phagocytosis results are represented by relative percentage to the group of cells incubated only with MSU crystals (100% \pm SEM). ANOVA followed by Newman-Keuls posttest and Student t test for graphic (A). * $p < 0.05$ compared with untreated cells. # $p < 0.05$ compared with cells stimulated with MSU crystals.

Again, this phenotype was also observed in neutrophils. Fc γ R $^{-/-}$ neutrophils had decreased phagocytosis of MSU crystals compared with WT cells, whereas Fc γ RIIB $^{-/-}$ and Fc γ RIII $^{-/-}$ neutrophils showed a similar phagocytosis rate. However, the addition of rPTX3 increased the phagocytosis rate only in WT and Fc γ RIIB $^{-/-}$ neutrophils, keeping the control levels in Fc γ R $^{-/-}$ and Fc γ RIII $^{-/-}$ neutrophils (Supplemental Fig. 2A, 2B).

To evaluate the relevance of these receptors in gouty inflammation, Fc γ R $^{-/-}$, Fc γ RIIB $^{-/-}$, and Fc γ RIII $^{-/-}$ mice were injected with MSU crystals, and the inflammatory parameters were evaluated 15 h later. As shown in Fig. 6, IL-1 β , CXCL1, and neutrophils were reduced in Fc γ R $^{-/-}$ and Fc γ RIII $^{-/-}$ when compared with WT mice. In contrast, there was no significant alteration of these parameters between WT and Fc γ RIIB $^{-/-}$ (Fig. 6). Altogether, these results suggest the involvement of the stimulatory Fc γ R Fc γ RIII in mice in PTX3-induced MSU crystal phagocytosis and joint inflammation.

Discussion

Several mechanisms involved in the pathogenesis of gout and recognition of MSU crystals have been discovered, including MSU crystal-triggered inflammasome activation and the contribution of different molecules and cell types to joint inflammation, damage,

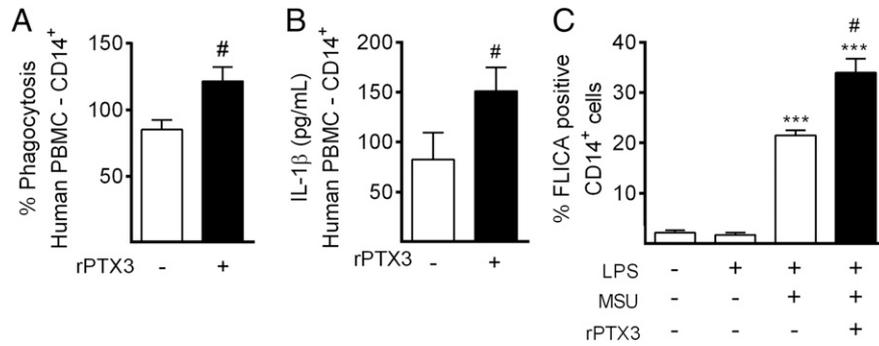


FIGURE 4. PTX3 facilitates the phagocytosis of MSU crystals and IL-β production by human monocytes. PBMC were incubated with MSU crystals (150 μg/ml) with or without rPTX3 (200 μg/ml), and the phagocytosis of the crystals was analyzed in CD14⁺ monocytes (A). Primed PBMC (LPS: 100 ng/ml; 1 h) were incubated with MSU crystals (150 μg/ml) for 6 h, and these crystals were pretreated or not with rPTX3 (200 μg/ml) for 1 h. The production of IL-1β (ELISA) in the supernatant (B) or FLICA-positive cells by flow cytometry, indicating caspase-1-positive cells (C) were determined in these cells. Results are mean ± SEM (n = 5–6). The experiments were repeated at least twice. Phagocytosis results are represented by relative percentage to the group of cells incubated only with MSU crystals (100% ± SEM). Mann–Whitney U test. ***p < 0.005 compared with untreated cells. #p < 0.05 compared with cells stimulated with MSU crystals.

and pain. This knowledge has potentially opened new possibilities for the therapy of gout. In the current study, we investigated the contribution of the soluble pattern recognition molecule PTX3 to gouty inflammation. The main results of this study can be summarized as follows: 1) high levels of PTX3 were found in plasma and synovial fluid of gouty patients during the acute phase of the disease with a positive correlation with IL-1β in the synovial fluid; 2) Ptx3-deficient mice showed diminished joint inflammation following the injection of MSU crystals; 3) PTX3 potentiated the phagocytosis of MSU crystals by human and murine cells, promoting an increase of inflammasome activation and IL-1β production; 4) the enhancement by PTX3 of the phagocytosis of MSU crystals and joint inflammation was dependent on FcγR.

PTX3 is an acute phase protein produced by different cells and body tissues. In the inflammatory context, a dual function of PTX3 has been proposed, either conferring host resistance against pathogens or contributing to immunopathogenesis of diseases (26). Circulating PTX3 is barely detectable in normal conditions but increases rapidly in a range of pathological conditions, including sepsis, *A. fumigatus* infections, and acute myocardial infarction (27–29). PTX3 is also expressed at high levels in synovial fluid during early joint inflammation in patients with systemic juvenile idiopathic arthritis (30). Moreover, synoviocytes and synovial fluid of RA patients displayed higher levels of PTX3 (17). Interestingly, a recent study demonstrated higher concentration of PTX3 in synovial fluid from RA patients testing positive for anticitrullinated protein Ab and rheumatoid factor when compared with seronegative patients. Moreover, there was a positive correlation between increased levels of (synovial fluid) PTX3 and the severity of the disease (31). In our population of gouty patients, we demonstrated an increase of PTX3 in plasma and synovial fluid during the acute phase when compared with healthy volunteers or OA patients. In addition, we showed a positive correlation between the levels of PTX3 and the amount of IL-1β in the synovial fluid of patients during the acute phase. Of note, the concentration of PTX3 was higher in synovial fluid than blood, suggesting local production of the molecule.

During the acute phase of gout, there is an elevated production and maturation of the cytokine IL-1β, a key agent for gout disease (4, 32). Because of relevance to gout, clinical trials with drugs that interfere on IL-1β–IL-1R pathway have been conducted, including rIL-1Ra (Anakinra), IL-1Trap (Rilonacept), and anti-IL-1β (Canakinumab), which provide rapid and sustained pain relief in patients with acute gouty arthritis and have revealed an impressive

and sustained reduction in recurrent attacks of gout (33–36). IL-1β is a potent inducer of PTX3 production and release by a variety of cell types, including mononuclear phagocytes, neutrophils, fibroblasts, endothelial cells, synovial cells, and chondrocytes (37), which are active cells during joint inflammation. Thus, the presence of PTX3 in the synovial fluid of gouty patients could represent an

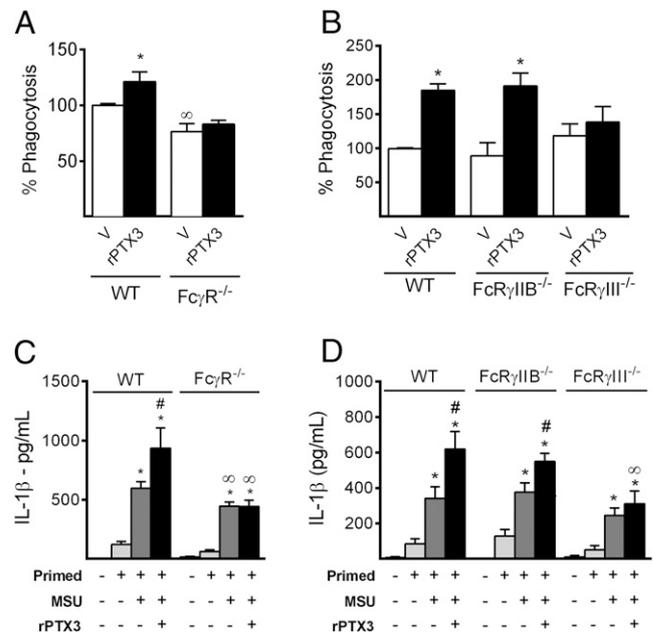


FIGURE 5. PTX3 facilitates the phagocytosis of MSU crystals in a mechanism dependent on FcγR, mainly FcγRIII. Whole blood from WT, FcγR^{-/-}, FcγRIIB^{-/-}, and FcγRIII^{-/-} mice was incubated with MSU crystals (150 μg/ml) during 15 min with or without rPTX3 (50 μg/ml), and the phagocytosis of the crystals was analyzed in CD45⁺CD11b⁺Ly6C^{high} monocytes (A and B). Primed peritoneal macrophages (LPS - 100 ng/ml; 1 h) were stimulated with MSU crystals (150 μg/ml) for 6 h to determine the amount of IL-1β by ELISA in the supernatant (C and D). Results are mean ± SEM (n = 3–7). The experiments were repeated at least two times. Phagocytosis results are represented by relative percentage to the group of cells incubated only with MSU crystals (100% ± SEM). ANOVA followed by Newman–Keuls posttest. t test to compare the levels of IL-1β between WT and FcγR^{-/-} in figure (C). *p < 0.05 compared with corresponding control cells. #p < 0.05 compared with cells stimulated with MSU crystals without rPTX3. §p < 0.05 compared with correlating WT groups. V, vehicle (culture media).

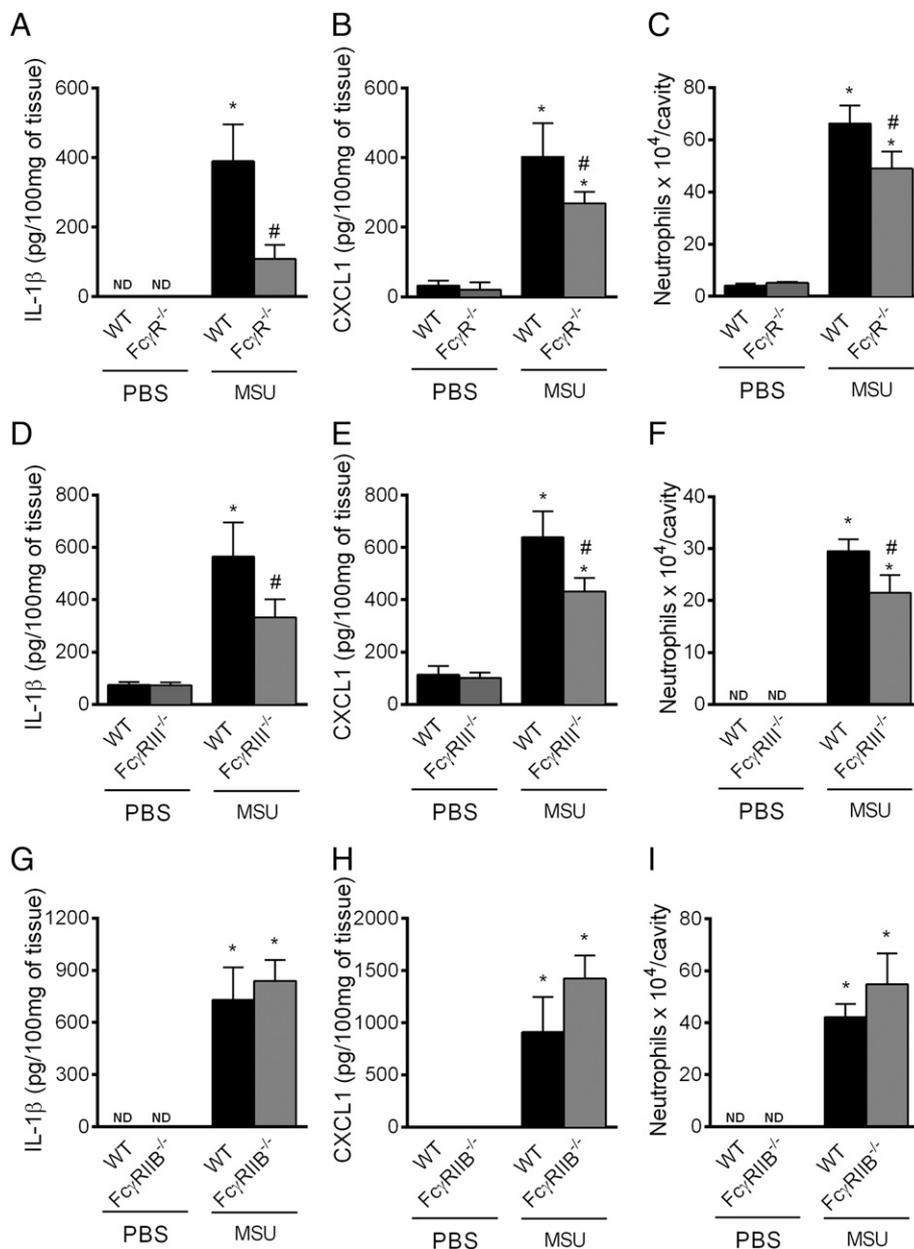


FIGURE 6. Stimulatory Fc γ R contributes to MSU crystal-induced joint inflammation. WT, Fc γ R^{-/-}, Fc γ RIIIB^{-/-}, and Fc γ RIII^{-/-} mice were injected with MSU crystals (100 μ g/cavity) into the tibiofemoral joint. Fifteen hours later, mice were culled for the quantification of IL-1 β (A, D, and G) and CXCL1 (B, E, and H) in the periarthritic tissue and neutrophil accumulation in the joint cavity (C, F, and I). Results are mean \pm SEM ($n = 4-8$). The experiments were repeated at least twice. ANOVA followed by Newman-Keuls. * $p < 0.05$ compared with PBS-injected mice. # $p < 0.05$ compared with WT mice injected with MSU crystals.

indirect marker of this disease or could have a direct contribution to joint inflammation. In this study, we demonstrated that PtX3-deficient mice have reduced inflammation following the injection of MSU crystals, suggesting an important role of this molecule in the development of gout.

The precise mechanisms by which innate immune molecules lead to the phagocytosis of MSU crystals are still not clear, although it is known that different serum factors can bind the crystal. MSU crystals isolated from sites of acute gouty inflammation are typically coated with Igs, mostly IgG isotype (5, 38). This interaction facilitates the engulfment of MSU crystals by macrophages and leads to the production of superoxide anion by phagocytes (39). There is evidence that MSU crystals interact with Fc γ RIIIB (CD16) in association with the CD11b/CD18 integrin complex in human neutrophils, whereas the receptor Fc γ RIIA (CD32) does not appear to play a direct role in mediating this effect (8). Importantly, Fc γ Rs have also been identified as pentraxins receptors (16). Moreover, it is known that both MSU crystals and PTX3 bind to C1q, the first subcomponent of the C1 complex of the classical pathway of complement activation (40, 41). In this way,

C1q could function as a bridge orchestrating the interaction between MSU crystals and PTX3, although further studies are necessary to clarify the nature of this interaction. It is known that PTX3 enhances phagocytosis of uropathogenic *Escherichia coli* (42) and *A. fumigatus* conidia (15) by human neutrophils. However, the contribution of PTX3 to MSU crystal phagocytosis and the involvement of Fc γ Rs in this process by mononuclear phagocytes had not yet been demonstrated. In this study, we demonstrated that PTX3 facilitates the phagocytosis of MSU crystals by both human and mouse monocytes and neutrophils in a mechanism dependent on Fc γ Rs in the murine context.

Humans and mice possess two classes of Fc γ Rs: the activating and inhibitory receptors based on their cytoplasmic tyrosine-containing motifs. Both species possess only one inhibitory Fc γ R, Fc γ RIIIB, which is expressed on B cells, dendritic cells, and basophils in both species, and found additionally on monocytes, macrophages, and all granulocyte populations in mice (43). In contrast, activating Fc γ Rs have remarkable differences between both species. Although murine-activating Fc γ Rs are represented by Fc γ RI, Fc γ RIII, and Fc γ RIV, the human activating Fc γ Rs are

composed by Fc γ RI, Fc γ RIIA, Fc γ RIIC, and Fc γ RIIIA (44). Based on the similarity of the extracellular portion sequence, murine Fc γ RIV is the ortholog of human Fc γ RIIIA, and murine Fc γ RIII is more closely related to human Fc γ RIIA; however, some differences between them are observed. Human Fc γ RIIA, for example, contains an intrinsic intracellular ITAM, whereas murine Fc γ RIII is noncovalently linked to the ITAM-containing Fc γ R adapter. In addition, there is an exclusive human receptor, Fc γ RIIIB, that is only expressed on neutrophils (45). Thus, the extrapolation of data regarding these receptors from animal studies to the human system must be very careful. In this study, cells obtained from mice deficient for Fc γ R had reduced phagocytosis when compared with WT cells. The addition of rPTX3 did not increase the engulfment of the crystals as it happened in WT cells. A similar profile was observed in murine Fc γ RIII, but not in Fc γ RIIIB-deficient cells, which responded equally to WT cells. However, cells from Fc γ R-deficient mice showed more pronounced response than Fc γ RIII-deficient cells, indicating that Fc γ RI and/or Fc γ RIV could also have a role in this process. Fc ϵ RI was not included in this study because it is the high-affinity receptor for the Fc region of IgE, which is not related to the MSU crystal-induced inflammation. Thus, our results clearly show the dependence of Fc γ R and specifically Fc γ RIII to enhancement of phagocytosis of MSU crystals by PTX3. The relevance of specific human Fc γ R classes in this context should be studied further in the future.

In conclusion, the results reported in this study indicate that PTX3 plays an important role in gouty inflammation by promoting the phagocytosis of MSU crystals via Fc γ Rs that culminates in increase of caspase-1 activation and IL-1 β secretion. PTX3 seems to be a very important mediator in the initial process of inflammation in gout and could be a target to control excessive inflammation induced by the deposition of MSU crystals in the joint.

Disclosures

The authors have no financial conflicts of interest.

References

- Kuo, C. F., M. J. Grainge, W. Zhang, and M. Doherty. 2015. Global epidemiology of gout: prevalence, incidence and risk factors. *Nat. Rev. Rheumatol.* 11: 649–662.
- Fam, A. G. 2005. Gout: excess calories, purines, and alcohol intake and beyond. Response to a urate-lowering diet. *J. Rheumatol.* 32: 773–777.
- Dalbeth, N., and D. O. Haskard. 2005. Mechanisms of inflammation in gout. *Rheumatology (Oxford)* 44: 1090–1096.
- Martinson, F., V. Pétrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237–241.
- Ortiz-Bravo, E., M. S. Sieck, and H. R. Schumacher, Jr. 1993. Changes in the proteins coating monosodium urate crystals during active and subsiding inflammation. Immunogold studies of synovial fluid from patients with gout and of fluid obtained using the rat subcutaneous air pouch model. *Arthritis Rheum.* 36: 1274–1285.
- Tramontini, N., C. Huber, R. Liu-Bryan, R. A. Terkeltaub, and K. S. Kilgore. 2004. Central role of complement membrane attack complex in monosodium urate crystal-induced neutrophilic rabbit knee synovitis. *Arthritis Rheum.* 50: 2633–2639.
- Russell, I. J., C. Papaioannou, F. C. McDuffie, S. MacIntyre, and I. Kushner. 1983. Effect of IgG and C-reactive protein on complement depletion by monosodium urate crystals. *J. Rheumatol.* 10: 425–433.
- Barabé, F., C. Gilbert, N. Liao, S. G. Bourgoin, and P. H. Naccache. 1998. Crystal-induced neutrophil activation VI. Involvement of Fc γ RIIIB (CD16) and CD11b in response to inflammatory microcrystals. *FASEB J.* 12: 209–220.
- Bottazzi, B., A. Doni, C. Garlanda, and A. Mantovani. 2010. An integrated view of humoral innate immunity: pentraxins as a paradigm. *Annu. Rev. Immunol.* 28: 157–183.
- Breviaro, F., E. M. d'Aniello, J. Golay, G. Peri, B. Bottazzi, A. Bairoch, S. Saccone, R. Marzella, V. Predazzi, M. Rocchi, et al. 1992. Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. *J. Biol. Chem.* 267: 22190–22197.
- Alles, V. V., B. Bottazzi, G. Peri, J. Golay, M. Introna, and A. Mantovani. 1994. Inducible expression of PTX3, a new member of the pentraxin family, in human mononuclear phagocytes. *Blood* 84: 3483–3493.
- Garlanda, C., B. Bottazzi, A. Bastone, and A. Mantovani. 2005. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu. Rev. Immunol.* 23: 337–366.
- Garlanda, C., E. Hirsch, S. Bozza, A. Salustri, M. De Acetis, R. Nota, A. Maccagno, F. Riva, B. Bottazzi, G. Peri, et al. 2002. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* 420: 182–186.
- Jaillon, S., G. Peri, Y. Delneste, I. Frémaux, A. Doni, F. Moalli, C. Garlanda, L. Romani, H. Gascan, S. Bellocchio, et al. 2007. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J. Exp. Med.* 204: 793–804.
- Moalli, F., A. Doni, L. Deban, T. Zelante, S. Zagarella, B. Bottazzi, L. Romani, A. Mantovani, and C. Garlanda. 2010. Role of complement and Fc γ receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus*. *Blood* 116: 5170–5180.
- Lu, J., L. L. Marnell, K. D. Marjon, C. Mold, T. W. Du Clos, and P. D. Sun. 2008. Structural recognition and functional activation of Fc γ RIIIb by innate pentraxins. *Nature* 456: 989–992.
- Luchetti, M. M., G. Piccinini, A. Mantovani, G. Peri, C. Matteucci, G. Pomponio, M. Fratini, P. Fraticelli, P. Sambo, C. Di Loreto, et al. 2000. Expression and production of the long pentraxin PTX3 in rheumatoid arthritis (RA). *Clin. Exp. Immunol.* 119: 196–202.
- Souza, D. G., F. A. Amaral, C. T. Fagundes, F. M. Coelho, R. M. Arantes, L. P. Sousa, M. M. Matzuk, C. Garlanda, A. Mantovani, A. A. Dias, and M. M. Teixeira. 2009. The long pentraxin PTX3 is crucial for tissue inflammation after intestinal ischemia and reperfusion in mice. *Am. J. Pathol.* 174: 1309–1318.
- Chen, C. J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K. L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J. Clin. Invest.* 116: 2262–2271.
- Amaral, F. A., V. V. Costa, L. D. Tavares, D. Sachs, F. M. Coelho, C. T. Fagundes, F. M. Soriani, T. N. Silveira, L. D. Cunha, D. S. Zamboni, et al. 2012. NLRP3 inflammasome-mediated neutrophil recruitment and hypernociception depend on leukotriene B(4) in a murine model of gout. *Arthritis Rheum.* 64: 474–484.
- Coelho, F. M., V. Pinho, F. A. Amaral, D. Sachs, V. V. Costa, D. H. Rodrigues, A. T. Vieira, T. A. Silva, D. G. Souza, R. Bertini, et al. 2008. The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature. *Arthritis Rheum.* 58: 2329–2337.
- Schorn, C., C. Janko, M. Latzko, R. Chaurio, G. Schett, and M. Herrmann. 2012. Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. *Front. Immunol.* 3: 277.
- Schroder, K., R. Zhou, and J. Tschopp. 2010. The NLRP3 inflammasome: a sensor for metabolic danger? *Science* 327: 296–300.
- Schroder, K., and J. Tschopp. 2010. The inflammasomes. *Cell* 140: 821–832.
- Tschopp, J., and K. Schroder. 2010. NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? *Nat. Rev. Immunol.* 10: 210–215.
- Magrini, E., A. Mantovani, and C. Garlanda. 2016. The dual complexity of PTX3 in health and disease: a balancing act? *Trends Mol. Med.* 22: 497–510.
- Uusitalo-Seppälä, R., R. Huttunen, J. Aittoniemi, P. Koskinen, A. Leino, T. Vahlberg, and E. M. Rintala. 2013. Pentraxin 3 (PTX3) is associated with severe sepsis and fatal disease in emergency room patients with suspected infection: a prospective cohort study. *PLoS One* 8: e53661.
- Salvatori, G., and S. Campo. 2012. Current understanding of PTX3 protective activity on *Aspergillus fumigatus* infection. *Med. Mycol.* 50: 225–233.
- Latini, R., A. P. Maggioni, G. Peri, L. Gonzini, D. Lucci, P. Mocarelli, L. Vago, F. Pasqualini, S. Signorini, D. Soldateschi, et al; Lipid Assessment Trial Italian Network (LATIN) Investigators. 2004. Prognostic significance of the long pentraxin PTX3 in acute myocardial infarction. *Circulation* 110: 2349–2354.
- Padeh, S., N. Farzam, G. Chayen, M. Gerstein, and Y. Berkun. 2013. Pentraxin 3 is a marker of early joint inflammation in patients with juvenile idiopathic arthritis. *Immunol. Res.* 56: 444–450.
- Weitof, T., A. Larsson, T. Saxne, V. A. Manivel, J. Lysholm, A. Knight, and J. Ronnelid. 2016. Pentraxin 3 in serum and synovial fluid of patients with rheumatoid arthritis with and without autoantibodies. *Scand. J. Rheumatol.* 46: 346–352.
- Torres, R., L. Macdonald, S. D. Croll, J. Reinhardt, A. Dore, S. Stevens, D. M. Hylton, J. S. Rudge, R. Liu-Bryan, R. A. Terkeltaub, et al. 2009. Hyperalgesia, synovitis and multiple biomarkers of inflammation are suppressed by interleukin 1 inhibition in a novel animal model of gouty arthritis. *Ann. Rheum. Dis.* 68: 1602–1608.
- Mitroulis, I., P. Skendros, and K. Ritis. 2010. Targeting IL-1 β in disease: the expanding role of NLRP3 inflammasome. *Eur. J. Intern. Med.* 21: 157–163.
- McGonagle, D., A. L. Tan, S. Shankaranarayanan, J. Madden, P. Emery, and M. F. McDermott. 2007. Management of treatment resistant inflammation of acute on chronic tophaceous gout with anakinra. *Ann. Rheum. Dis.* 66: 1683–1684.
- Sundy, J. S., H. R. Schumacher, A. Kivitz, S. P. Weinstein, R. Wu, S. King-Davis, and R. R. Evans. 2014. Rilonecept for gout flare prevention in patients receiving uric acid-lowering therapy: results of RESURGE, a phase III, international safety study. *J. Rheumatol.* 41: 1703–1711.
- Schlesinger, N. 2012. Canakinumab in gout. *Expert Opin. Biol. Ther.* 12: 1265–1275.
- Mantovani, A., C. Garlanda, A. Doni, and B. Bottazzi. 2008. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. *J. Clin. Immunol.* 28: 1–13.

38. Cherian, P. V., and H. R. Schumacher, Jr. 1986. Immunochemical and ultrastructural characterization of serum proteins associated with monosodium urate crystals (MSU) in synovial fluid cells from patients with gout. *Ultrastruct. Pathol.* 10: 209–219.
39. Nagase, M., D. G. Baker, and H. R. Schumacher, Jr. 1989. Immunoglobulin G coating on crystals and ceramics enhances polymorphonuclear cell superoxide production: correlation with immunoglobulin G adsorbed. *J. Rheumatol.* 16: 971–976.
40. Hasselbacher, P. 1979. Binding of IgG and complement protein by monosodium urate monohydrate and other crystals. *J. Lab. Clin. Med.* 94: 532–541.
41. Nauta, A. J., B. Bottazzi, A. Mantovani, G. Salvatori, U. Kishore, W. J. Schwaeble, A. R. Gingras, S. Tzima, F. Vivanco, J. Egido, et al. 2003. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *Eur. J. Immunol.* 33: 465–473.
42. Jaillon, S., F. Moalli, B. Ragnarsdottir, E. Bonavita, M. Puthia, F. Riva, E. Barbati, M. Nebuloni, L. Cvetko Krajcinovic, A. Markotic, et al. 2014. The humoral pattern recognition molecule PTX3 is a key component of innate immunity against urinary tract infection. *Immunity* 40: 621–632.
43. Powell, M. S., and P. M. Hogarth. 2008. Fc receptors. *Adv. Exp. Med. Biol.* 640: 22–34.
44. Hargreaves, C. E., M. J. Rose-Zerilli, L. R. Machado, C. Iriyama, E. J. Hollox, M. S. Cragg, and J. C. Strefford. 2015. Fcγ receptors: genetic variation, function, and disease. *Immunol. Rev.* 268: 6–24.
45. Futosi, K., S. Fodor, and A. Mócsai. 2013. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* 17: 638–650.