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The long pentraxin PTX3 contributes to joint inflammation in gout by facilitating the phagocytosis of MSU crystals

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Abstract

Objective—We aimed to investigate the role of pentraxin 3 (PTX3), a pivotal component of the innate immune system, in gout.

Methods—Two different cohorts of gout patients were investigated. A murine model of acute gout was induced by injection of monosodium urate (MSU) crystals in wild type mice and in Ptx3-, FcγR-, FcγRIIB-, and FcγRIII-deficient mice. PTX3 and IL-1β were quantified by ELISA in synovial fluid and plasma of patients. PTX3, IL-1β, CXCL1 and neutrophil recruitment were evaluated in inflamed murine joint. The phagocytosis of MSU crystals and caspase-1 activation were determined in isolated cells by flow cytometer.

Results—Acute gout patients showed elevated concentration of PTX3 in plasma and synovial fluid as compared to healthy and osteoarthritic subjects. Moreover, there was a positive correlation between intra-articular PTX3 and IL-1β levels. PTX3 was induced in the periarticular tissue of mice after injection of MSU crystals. Importantly, Ptx3-deficient mice showed reduced inflammation in response to MSU crystals compared to WT mice, including reduction of neutrophil recruitment into the joint cavity, and IL-1β and CXCL1 production. Interestingly, addition of PTX3 *in vitro* enhanced MSU crystal phagocytosis by monocytes and resulted in

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higher caspase-1 activation and production of IL-1β by macrophages. This contribution of PTX3 to the phagocytosis of MSU crystals and consequent production of IL-1β occurred through a mechanism mainly dependent on stimulatory FcγR.

Conclusion—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.

Keywords

PTX3; gout; arthritis; inflammasome

Introduction

Gout is the most common form of inflammatory arthritis worldwide among men and post-menopausal women[12]. Joint inflammation in gout occurs due to the deposition of monosodium urate (MSU) crystals predominantly in peripheral joints and surrounding tissues, and mainly in individuals with chronic hyperuricemia. Acute gout attacks 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 subcutaneous tophi[3].

The initial events of MSU crystals-triggered inflammation occur after the contact and phagocytosis of MSU crystals by synovial fluid phagocytes, leading the assembly of NLRP3/ASC/caspase-1 inflammasome that culminates in the release of the mature form of IL-1β[4], a key cytokine in gouty arthritis. IL-1β 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 and FcγRIIIB, which 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 antibodies and pentraxins (PTXs) constitute the humoral arm of the innate immune response[9]. Pentraxins are a superfamily of evolutionarily conserved multimeric proteins, which is divided into short 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 lipopolysaccharide (LPS), IL-1 and TNF-α[10–12]. PTX3 can interact with a variety of pathogens and has opsonic activity facilitating their phagocytosis[13–15] through interaction with Fcγ receptors (FcγR), which have been identified as pentraxin receptors[16]. Moreover, PTX3 is involved in the pathogenesis of acute and chronic sterile inflammatory diseases, including ischemia/reperfusion and rheumatoid arthritis[1718]. However, the involvement of

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PTX3 in gout has not yet been described. The present study was designed to investigate the role of PTX3 in gouty inflammation.

Materials and Methods

Samples of patients diagnosed with gout

Two Brazilian cohorts of patients diagnosed with acute gout flares, according to the 2015 Gout Classification Criteria, were used in this study: from Rio de Janeiro (A – 8 patients) and from Ribeirão Preto (B – 19 patients). One cohort of patients diagnosed with osteoarthritis of the knee (Ribeirão Preto – 12 patients), according to the Classification of American Rheumatism Association[19], was used in this study. Synovial fluid and blood samples were collected in EDTA tubes, centrifuged (2,000g-10min) and the supernatant kept at -20°C. A fresh sample of synovial fluids from gout and osteoarthritis underwent MSU crystals identification by compensated polarized light microscopy. All patients provided informed consent to participate the study, which was approved by CEP under protocol number 1.297.041, and by CAAE (Conep) 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 background. Italy: Ptx3-deficient mice were generated as described[13]. FcRγ-deficient mice were purchased from The Jackson Labs, Bar Harbor ME/USA. All colonies were housed and bred in the SPF animal facility of Humanitas Clinical and Research Center. 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 (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 2010/63/EU, OJ 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 n. 71/2012-B). Brazil: FcγRIIB- and FcγRIII-deficient mice were a kind gift of Dr. Josef 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-CETEA/UFMG).

Experimental model of acute gout

Mice were placed under anesthesia (80:15mg/kg ketamine:xylazine, i.p.; Syntec/Brazil) and were injected with MSU crystals (100μg/cavity) into the tibiofemoral joint. MSU crystals were prepared as previously described[20]. Inflammatory parameters were evaluated at 6 or 15 hours after injection of MSU crystals. Mice were euthanized by anaesthesia followed by cervical dislocation, and the articular cavity was washed with PBS containing 3% bovine serum albumin for leukocyte counts. The number of cells was determined in a Neubauer

chamber after staining with Turk's solution. Differential counts were performed by Shandon CytoSpin III (Thermo Shandon, Frankfurt, Germany) preparations by evaluating the percentage of each leukocyte after staining with May Grunwald-Giemsa[21].

ELISA

Periarticular tissues were collected from joints and homogenized with PBS containing antiproteases[22]. Samples were centrifuged, and the supernatant was used to quantify PTX3, IL-1β and CXCL1. Plasma and synovial fluid from patients were used for determination of PTX3 and IL-1β. A commercially available ELISA kit was used in accordance with the manufacturer's instructions (R&D Systems/USA).

Phagocytosis of MSU crystals assay

One hundred microliters of mouse whole blood were incubated with MSU crystals (150μg/mL) for 15 minutes, at 37°C. Previously, crystals were incubated or not with recombinant PTX3 (rPTX3) for 1 hour. We tested different concentrations of rPTX3 and presented those results which had significance compared to the groups without rPTX3. Human cells were isolated with a density gradient centrifugation in accordance with the manufacturer's instructions (Histopaque1077/1119 - Sigma-Aldrich/USA). PBMC (peripheral blood mononuclear cell) were plated for 2 hours to allow the adherence and then these cells or isolated neutrophils ($5x10^5$) were stimulated as murine cells described above. Purity was checked using cytospin preparation and all samples used exceeded 90% purity. After stimulation, samples were placed on ice to block phagocytosis, and red cells were lysed by adding cold ammonium chloride lysis solution pH 7.2. As control, cells were pre-incubated with 2.5μM of cytochalasin D (Sigma-Aldrich/USA) for 1 hour before the crystals. Samples were stained for identification of monocytes and neutrophils by cell specific surface markers: CD14 (monocytes) for human and CD45-CD11b-LyG (neutrophils) and Ly6C (monocytes) for mouse (eBiosciences, BD Pharmingen). Samples were then fixed with 1% paraformaldehyde. The phagocytosis was determined by analysis of SSC change in flow cytometry[23]. 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 (100ng/mL) for 1 hour and then stimulated with MSU crystals (150μg/mL) for 6 hours. Cells were stained with FLICA in accordance with the manufacturer's instructions and for CD14 for identification of monocytes. The analyses were performed with FlowJo software (Tree Star).

Preparation and culture of murine macrophages

Mice received an intraperitoneal injection of a 3% thioglycolate solution and macrophages were collected by peritoneal lavage 4 days later. Cells were plated for 2 hours and nonadherent cells were removed. Cells were cultured in RPMI medium complemented with

10% fetal calf serum, penicillin/streptomycin, and L-glutamine, and kept in a humidified incubator at 37°C with 5% CO_2 overnight. Cells were primed for 1 hour with LPS (100ng/ml) (Sigma-Aldrich/USA) and then stimulated with MSU crystals (150μg/ml) for 6 hours. The supernatant was used for IL-1β detection by ELISA.

Statistical analysis

All results are presented as the means±standard error of mean (SEM). Results were tested for normality and differences between groups were evaluated using one-way ANOVA followed by Newman-Keuls post-test, Student's t test, Whitney U test or Pearson's correlation as specified. The level of significance was set at P values less than 0.05. Statistics were calculated with GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA/USA).

Results

PTX3 positively correlates with IL-1β 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 cohort A, there was an increase of PTX3 levels in plasma of patients with gout compared to healthy volunteers. Interestingly, the levels of PTX3 were higher in synovial fluid as compared to plasma in those patients (Figure 1A). In cohort B, the concentration of PTX3 in synovial fluid was much higher in gout patients compared to OA patients (Figure 1B). In both cohorts, IL-1β was detected in synovial fluid of gouty patients (data not shown). Importantly, there was a positive correlation between PTX3 and IL-1β in synovial fluid during acute gout in cohort A ($r = 0.52$; $p = 0.04$) and cohort B (Figure 1C). However, there was no positive correlation between PTX3 and IL-1β in plasma of cohort A (data not shown).

PTX3 contributes to gouty inflammation

Next, we used an experimental model of acute gout in mice [21]. Injection of MSU crystals caused an increase of PTX3 levels in inflamed joint 6 hours after the injection when compared to saline-injected joint and this increase was even greater at the 15th hour (Figure 2A). MSU crystals also caused an increase of IL-1β, CXCL1 and influx of neutrophils to the joint compared to control group (Figure 2B-D). Ptx3-deficient mice had a reduction in these inflammatory parameters following the injection of MSU crystals as compared to WT mice (Figure 2B-D). Altogether, these data strengthen the contribution of PTX3 to MSU crystals-induced inflammation.

PTX3 facilitates the phagocytosis of MSU crystals and cell activation

PTX3 opsonizes different microorganisms contributing their pathogen phagocytosis and clearance[13–15]. Here, we first treated murine whole blood with cytochalasin D (CytD), an inhibitor of actin polymerization that impairs phagocytosis, before MSU crystal stimulation and performed the analysis of phagocytosis by flow cytometry. $CD45^{+}CD11b^{+}Ly6C^{high}$ monocytes phagocytosed MSU crystals and this was significantly reduced after CytD treatment (Figure 3A). MSU crystals stimulated the release of IL-1β by LPS-primed peritoneal macrophages. Incubation with CytD showed decreased the release of IL-1β levels

in the supernatant, confirming that phagocytosis of MSU crystals is necessary for IL-1β maturation and release (Figure 3B). Ptx3-deficient monocytes showed defective phagocytic activity when compared to 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 (Figure 3C). Peritoneal macrophages incubated with rPTX3 also increased the release of IL-1β after MSU crystals stimulation in both WT and Ptx3-deficient cells (Figure 3D). $CD45^{+}CD11b^{high}Ly6G^{high}$ neutrophils from Ptx3-deficient mice had reduced phagocytosis of MSU crystals when compared to WT neutrophils, a phenotype that was rescued by the addition of rPTX3 (Supplementary Figure 1A).

The presence of rPTX3 also increased the phagocytosis of MSU crystals by human blood $CD14^{+}$ monocytes from healthy donors (Figure 4A) and lead to increase of IL-1β in the supernatant (Figure 4B). In addition, the incubation with rPTX3 increased MSU crystal-dependent caspase-1 activation when compared to cells treated only with MSU crystals (Figure 4C). Along the same line, isolated human neutrophils incubated with MSU crystals in the presence of rPTX3 showed greater phagocytosis (Supplementary Figure 1C). Thus, PTX3 facilitates the phagocytosis of MSU crystals and consequently favours the production of IL-1β in mononuclear phagocytes.

The role of PTX3 in gout depends on FcγRs

PTX3 mediates part of its biological activities by the interaction with FcγRs[16]. Monocytes from Fc common γ-chain receptors (FcγR)-deficient mice had lower phagocytosis rate in comparison to cells from WT mice. Interestingly, the addition of rPTX3 to MSU crystals-stimulated $Fc\gamma R^{-/-}$ cells did not increase the phagocytosis of the crystals, in contrast with what observed with WT cells (Figure 5A). Next, we tested this response in cells from FcγRIIB (an inhibitory Fcγ receptor)-deficient and FcγRIII (a stimulatory Fcγ receptor)-deficient mice. Monocytes from both $Fc\gamma RIIB^{-/-}$ and $Fc\gamma RIII^{-/-}$ mice showed the same levels of phagocytosis of MSU crystals as WT mice. However, cells from WT and $Fc\gamma RIIB^{-/-}$, but not from $Fc\gamma RIII^{-/-}$ mice, showed increased phagocytosis after the incubation with rPTX3 (Figure 5B). We found a similar profile when analysing IL-1β release. WT, $Fc\gamma R^{-/-}$, $Fc\gamma RIIB^{-/-}$ and $Fc\gamma RIII^{-/-}$ primed macrophages stimulated with MSU crystals released more IL-1β levels in the supernatant than non-stimulated cells. However, the incubation with rPTX3 increased the amount of IL-1β further only in WT and $Fc\gamma RIIB^{-/-}$ cells, but did not affect IL-1β release by $Fc\gamma R^{-/-}$ and $Fc\gamma RIII^{-/-}$ cells (Figure 5C,D).

This phenotype was also observed in neutrophils. $Fc\gamma R^{-/-}$ neutrophils had decreased phagocytosis of MSU crystals compared to WT cells, whereas $Fc\gamma RIIB^{-/-}$ and $Fc\gamma RIII^{-/-}$ neutrophils showed a similar phagocytosis rate. However, the addition of rPTX3 increased the phagocytosis rate only in WT and $Fc\gamma RIIB^{-/-}$ neutrophils, keeping the control levels in $Fc\gamma R^{-/-}$ and $Fc\gamma RIII^{-/-}$ neutrophils (Supplementary Figure 1A,B).

In order to evaluate the relevance of these receptors in gouty inflammation, $Fc\gamma R^{-/-}$, $Fc\gamma RIIB^{-/-}$ and $Fc\gamma RIII^{-/-}$ mice were injected with MSU crystals and the inflammatory parameters were evaluated 15 hours later. As shown in Figure 6, IL-1β, CXCL1 and neutrophils were reduced in $Fc\gamma R^{-/-}$ and $Fc\gamma RIII^{-/-}$ when compared to WT mice. In contrast,

there was no significant alteration of these parameters between WT and FcγRIIB$^{-/-}$ (Figure 6).

Discussion

Circulating PTX3, an acute phase protein, is barely detectable in normal conditions but increases rapidly in a range of pathological conditions, including sepsis and acute myocardial infarction[24–26]. PTX3 is also expressed at high levels in synovial fluid during early joint inflammation in patients with systemic juvenile idiopathic arthritis[27]. Moreover, synoviocytes and synovial fluid of rheumatoid arthritis (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 anti-citrullinated protein antibody and rheumatoid factor when compared to seronegative patients. Moreover, there was a positive correlation between increased levels of PTX3 and the severity of the disease[28]. In our population of gouty patients, we demonstrated an increase of PTX3 in plasma and synovial fluid during the acute phase when compared to healthy volunteers or OA patients. In addition, we showed a positive correlation between the levels of PTX3 and 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 was an elevated production of IL-1β, a key agent for gout pathology[429]. Due to its relevance to gout, clinical trials with drugs that interfere on IL-1β/IL-1R pathway have been conducted, including recombinant IL-1Ra, IL-1Trap and anti-IL-1β, which provide rapid and sustained pain relief in patients with acute gout, and have revealed an impressive and sustained reduction in recurrent attacks [30–33]. IL-1β is a potent inducer of PTX3 production and release by a variety of cell types, including mononuclear phagocytes and neutrophils[34], which are active cells during joint inflammation. Thus, the presence of PTX3 in the synovial fluid of gouty patients could represent an indirect marker of this disease or could have a direct contribution to joint inflammation.

MSU crystals isolated from sites of acute gouty inflammation are typically coated with immunoglobulins[535]. This interaction facilitates the engulfment of MSU crystals and leads to the production of superoxide anion by phagocytes[36]. There is evidence that MSU crystals interact with FcγRIIIB in association with the CD11b/CD18 integrin complex in human neutrophils, while the receptor FcγRIIA does not appear to play a direct role in mediating this effect[8]. Importantly, FcγRs 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[3738]. 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.

Humans and mice possess two classes of FcγRs, the activating and inhibitory receptors based on their cytoplasmic tyrosine-containing motifs. Both species possess only one inhibitory FcγR, FcγRIIB[39]. On the other hand, activating FcγRs have remarkable differences between both species. While murine activating FcγRs are represented by FcγRI, FcγRIII and FcγRIV, the human activating FcγRs are composed by FcγRI, FcγRIIA,

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FcγRIIC, FcγRIIIA[40]. Based on the similarity of the extracellular portion sequence, murine FcγRIV is the orthologue of human FcγRIIIA and murine FcγRIII is more closely related to human FcγRIIA, however some difference between them are observed. In addition, there is an exclusive human receptor, FcγRIIIB, that is only expressed on neutrophils[41]. 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 common γ-chain receptors (FcγR) or FcγRIII had reduced phagocytosis when compared to WT cells. The addition of rPTX3 did not increase the engulfment of the crystals as it happened in WT cells. However, cells from FcγR-deficient mice showed more pronounced response than FcγRIII-deficient cells, indicating that FcγRI and/or FcγRIV could also have a role in this process. FcεRI was not included here since it is the high-affinity receptor for the Fc region of immunoglobulin E which is not related to the MSU crystal-induced inflammation. The relevance of specific human FcγR classes in this context should be studied further in the future.

In conclusion, the results reported here indicate that PTX3 plays an important role in gouty inflammation by promoting the phagocytosis of MSU crystals via FcγRs that culminates in the increase of caspase-1 activation and IL-1β 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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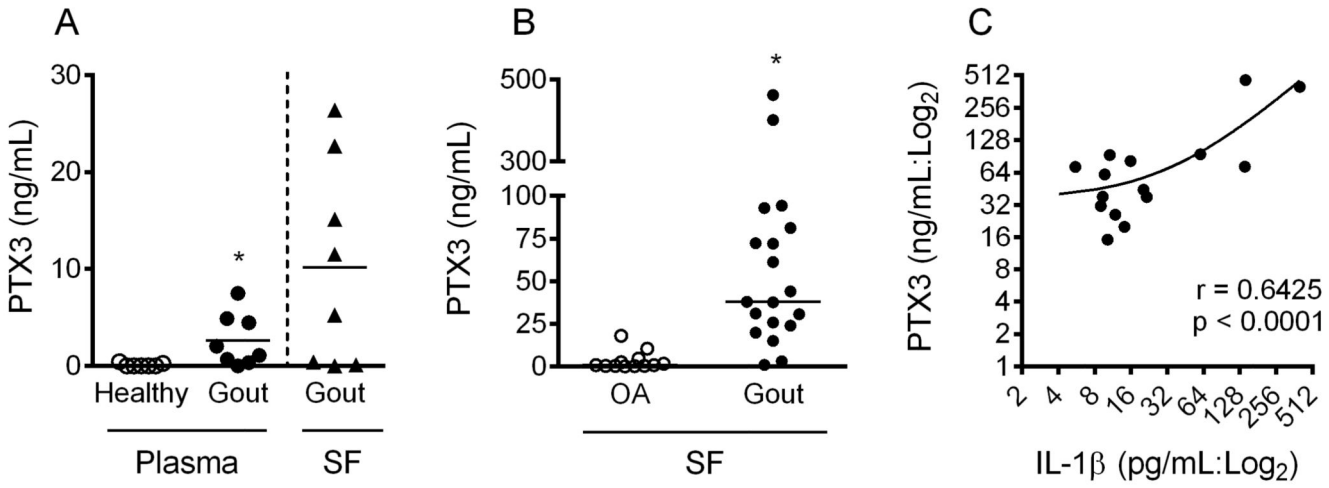

Figure 1. The amount of PTX3 is increased in gouty patients and positively correlated with IL-1β levels.

The amount of PTX3 was measured by ELISA in plasma and synovial fluid of healthy and gouty patients from cohort A (A). The amount of PTX3 was measured by ELISA in synovial fluid of osteoarthritic (OA) and gouty patients from cohort B (B). Pearson correlation analysis between PTX3 and IL-1β in synovial fluid obtained from cohort B (C). *$p<0.05$, compared to its control; Mann-Whitney U test or Pearson correlation for graphic C.

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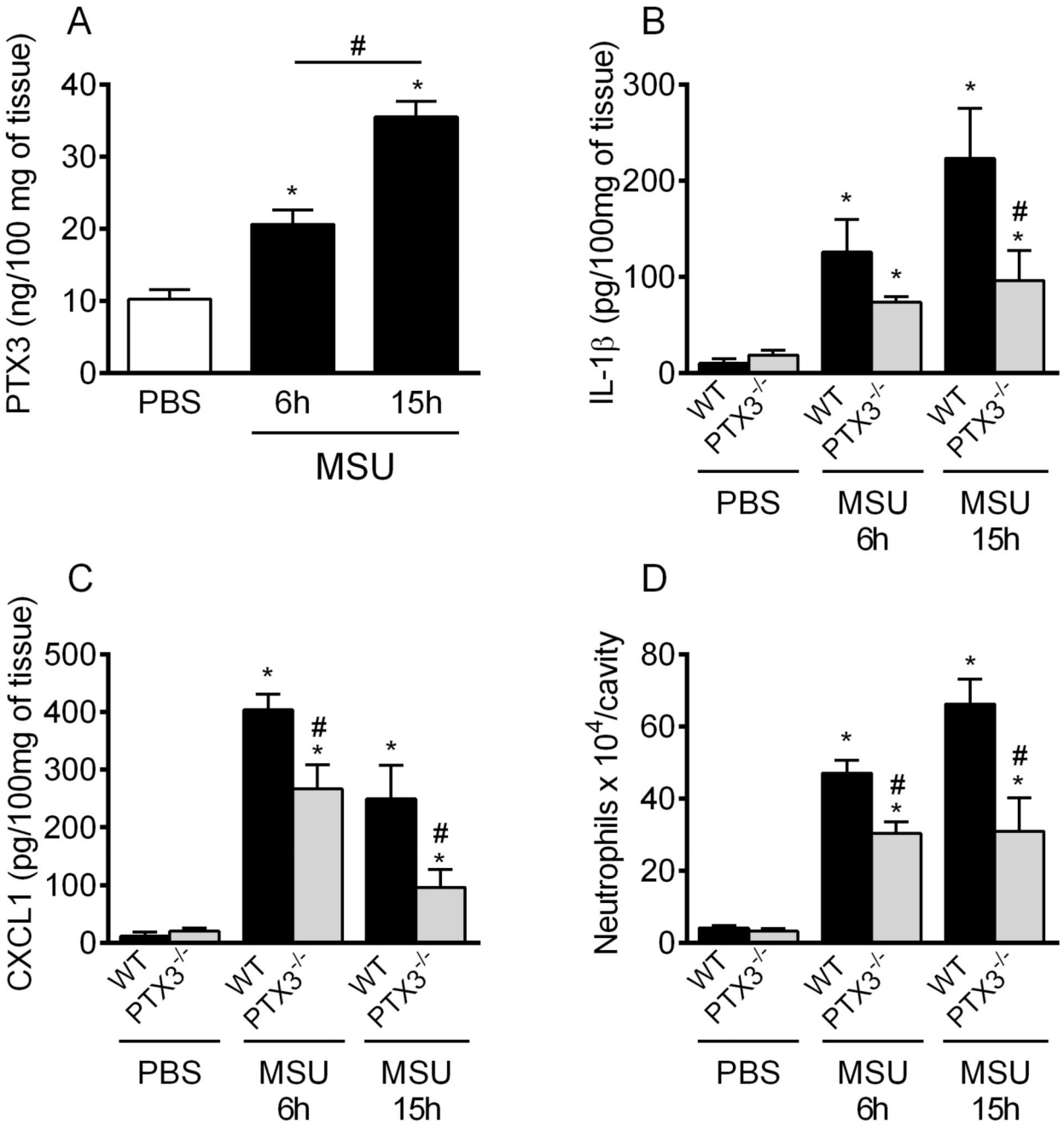

Figure 2. PTX3 participates in MSU crystal-induced joint inflammation.

Wild type (WT) mice received an intra-articular injection of MSU crystals (100 μg/cavity) and periarticular tissue was collected at different time points (6 or 15 hours after MSU crystal injection) to quantify PTX3 by ELISA (A). Concentrations of IL-1β (B) and CXCL1 (C) in homogenized periarticular tissue and the accumulation of neutrophils into the joint cavity (D) in wild-type and $Ptx3^{-/-}$ mice after 15 hours following the injection of MSU crystals. Results are presented as the mean ± SEM (n=5-6). The experiments were repeated at least twice. *$p<0.05$ compared to PBS-injected mice and #$p<0.05$ compared to MSU

crystals-injected WT mice corresponding to each time point. ANOVA followed by Newman-Keuls post-test.

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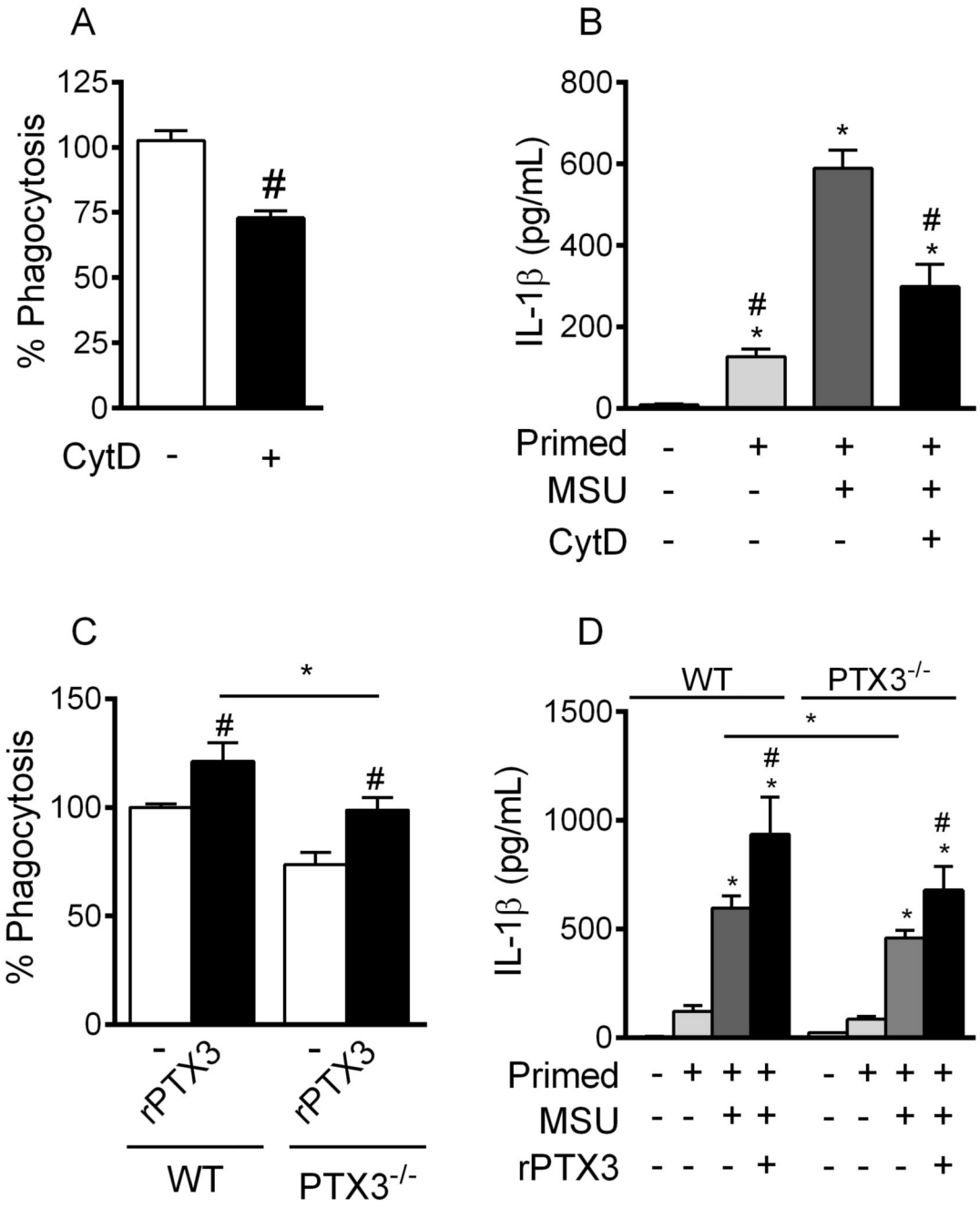

Figure 3. PTX3 increases the phagocytosis of MSU crystals and IL-1β production by mononuclear phagocyte-derived murine cells.

The phagocytosis of MSU crystals were evaluated in monocytes CD45+CD11b+ Ly6C^{high} after the incubation of whole blood with the crystals for 15 minutes and analysed by SSC change in flow cytometry (A and C). IL-1β was quantified by ELISA in the supernatant of peritoneal macrophages stimulated with MSU crystals for 6 hours (B and D). Cells from wild type mice (WT) were pre-treated with cytochalasin D (CytD – 2.5 μM) 1 hour before the stimulation with MSU crystals (A and B). Cells from WT and Ptx3$^{-/-}$ mice were stimulated with MSU crystals, preincubated or not with recombinant PTX3 (50 μg/mL), for

6 hours (C and D). For priming, peritoneal macrophages were incubated with LPS (100 ng/mL) for 1 hour before the incubation with MSU crystals. 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). *p<0.05 compared to untreated cells and #p<0.05 compared to cells stimulated with MSU crystals; ANOVA followed by Newman-Keuls post-test and Student's t test for graphic A.

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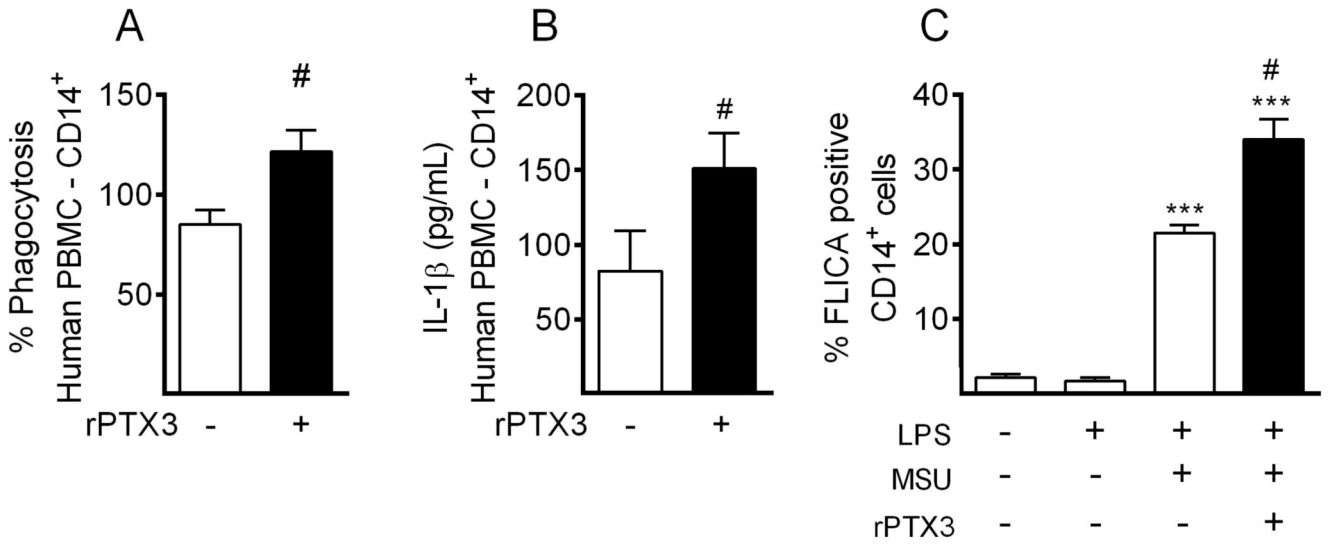

Figure 4. PTX3 facilitates the phagocytosis of MSU crystals and IL-β production by human monocytes.

PBMC were incubated with MSU crystals (150 μg/mL) with or without recombinant PTX3 (200 μg/mL) and the phagocytosis of the crystals was analyzed in $CD14^+$ monocytes (A). Primed PBMC (LPS - 100 ng/mL; 1 hour) were incubated with MSU crystals (150 μg/mL) for 6 hours and these crystals were pretreated or not with recombinant PTX3 (200 μg/mL) for 1 hour. 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). * $p<0.05$ compared to untreated cells #$p<0.05$ compared to cells stimulated with MSU crystals; Mann-Whitney U test.

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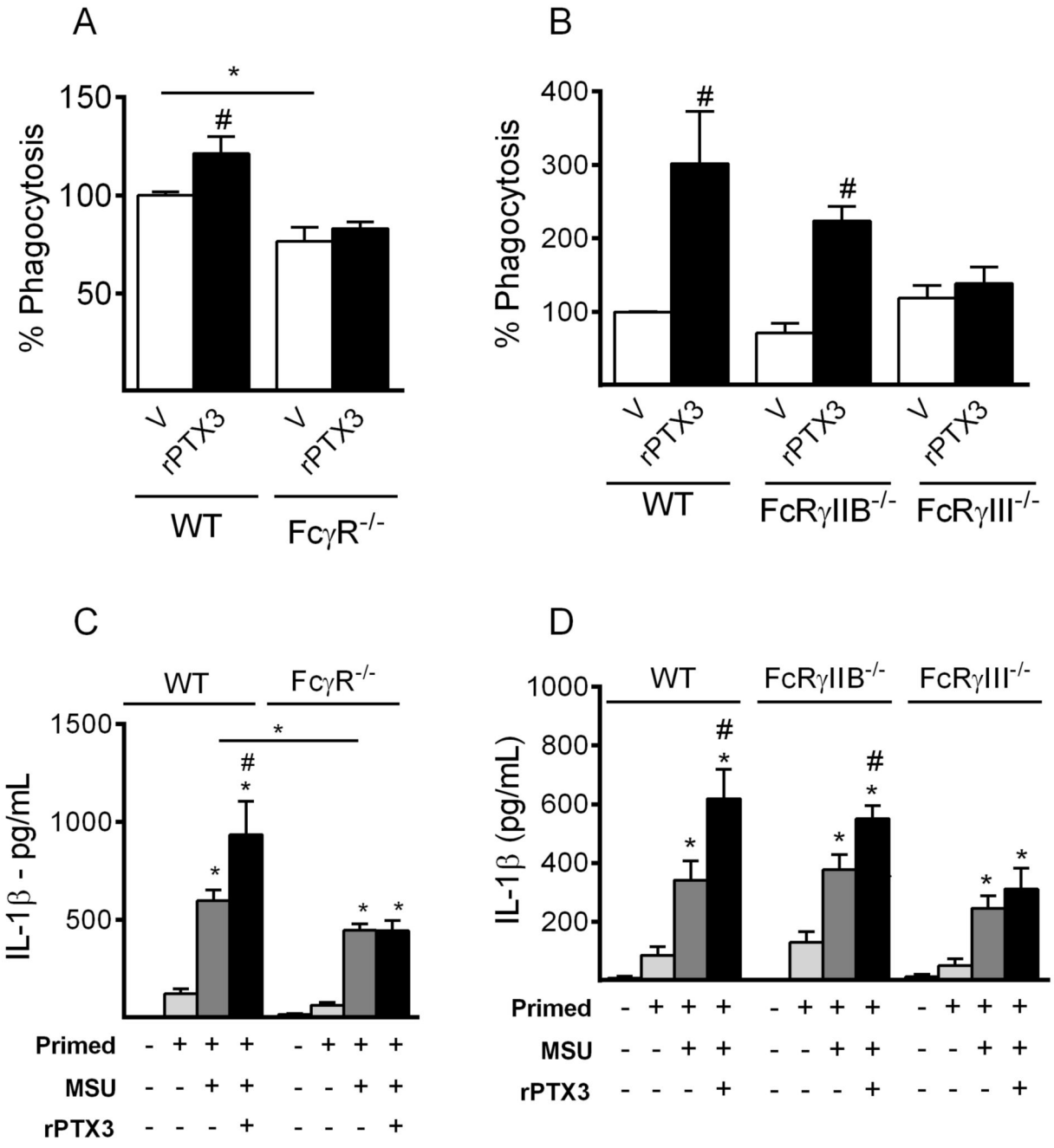

Figure 5. PTX3 facilitates the phagocytosis of MSU crystals in a mechanism dependent on FcγR, mainly FcγRIII.

Whole blood from wild-type (WT), FcγR$^{-/-}$, FcγRIIB$^{-/-}$ and FcγRIII$^{-/-}$ mice was incubated with MSU crystals (150 μg/mL) during 15 minutes with or without recombinant PTX3 (50 μg/mL) and the phagocytosis of the crystals was analysed in CD45^{+}CD11b^{+} Ly6C^{high} monocytes (A and B). Primed peritoneal macrophages (LPS - 100 ng/mL; 1 hour) were stimulated with MSU crystals (150 μg/mL) for 6 hours to determine the amount of IL-1β by ELISA in the supernatant (C and D). Results are mean ± SEM (n=5-6). 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). *p<0.05 compared to untreated cells and #p<0.05 compared to cells stimulated with MSU crystals; ANOVA followed by Newman-Keuls post-test.

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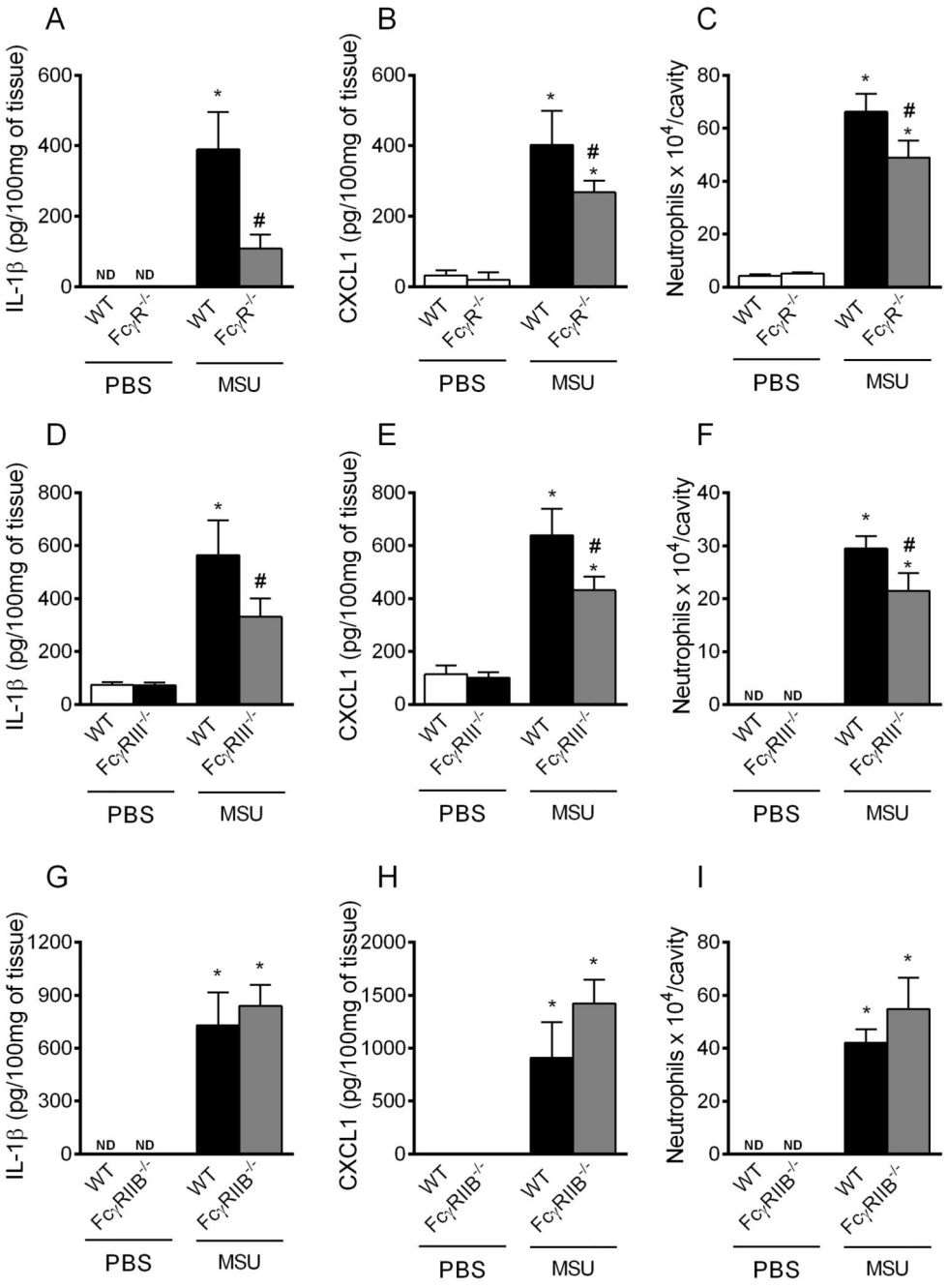

Figure 6. Stimulatory FcγR contributes to MSU crystal-induced joint inflammation.

Wild-type (WT), FcγR$^{-/-}$, FcγRIIB$^{-/-}$ and FcγRIII$^{-/-}$ mice were injected with MSU crystals (100 μg/cavity) into the tibiofemoral joint. Fifteen hours later, mice were culled for the quantification of IL-1β (A, D, G) and CXCL1 (B, E, H) in the periarticular tissue and neutrophil accumulation in the joint cavity (C, F, I). Results are mean ± SEM (n=4-8). The experiments were repeated at least twice. *p<0.05 compared to PBS-injected mice and

#p<0.05 compared to wild-type mice injected with MSU crystals; ANOVA followed by Newman-Keuls.

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