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# The peptidyl-prolyl isomerase Pin1 controls GM-CSF-induced priming of NADPH oxidase in human neutrophils and priming at inflammatory sites

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## ABSTRACT

The production of superoxide anions and other reactive oxygen species (ROS) by neutrophils is necessary for host defense against microbes. However, excessive ROS production can induce cell damage that participates in the inflammatory response. Superoxide anions are produced by the phagocyte NADPH oxidase, a multicomponent enzyme system consisting of two transmembrane proteins (gp91phox/NOX2 and p22phox) and four soluble cytosolic proteins (p40phox, p47phox, p67phox and the small G proteins Rac1/2). Stimulation of neutrophils by various agonists, such as the bacterial peptide formyl-Met-Leu-Phe (fMLF), induces NADPH oxidase activation and superoxide production, a process that is enhanced by the pro-inflammatory cytokines such as GM-CSF. The pathways involved in this GM-CSF-induced up-regulation or priming are not fully understood. Here we show that GM-CSF induces the activation of the prolyl *cis/trans* isomerase Pin1 in human neutrophils. Juglone and PiB, two selective Pin1 inhibitors, were able to block GM-CSF-induced priming of ROS production by human neutrophils. Interestingly, GM-CSF induced Pin1 binding to phosphorylated p47phox at Ser345. Neutrophils isolated from synovial fluid of patients with rheumatoid arthritis are known to be primed. Here we show that Pin1 activity was also increased in these neutrophils and that Pin1 inhibitors effectively inhibited ROS hyperproduction by the same cells. These results suggest that the prolyl *cis/trans* isomerase Pin1 may control GM-CSF-induced priming of ROS production by neutrophils and priming of neutrophils in synovial fluid of rheumatoid arthritis patients. Pharmacological targeting of Pin1 may be a valuable approach to the treatment of inflammation.

## 1. Introduction

Neutrophils are the most abundant leukocytes in human blood and are key cells of innate immunity and inflammatory response [1,2]. Upon infection, neutrophils are attracted by a variety of agents, including complement C5a, N-formyl-methionyl-leucyl-phenylalanine (fMLF), interleukin 8 (IL-8), LTB4, and platelet-activating factor (PAF), and are

the first circulating cells to reach the site of infection [1–4]. At the site of infection, they engulf the microbe and release large amounts of antibacterial agents such as reactive oxygen species (ROS), myeloperoxidase, proteases, glucosidases, and antibacterial peptides in order to kill and eliminate microbes [5,6]. However, excessive release of these toxic agents can induce tissue damage that may contribute to inflammation [7–10].

**Abbreviations:** fMLP or fMLF, N-formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage colony stimulating factor; HBSS, Hanks' Balanced Salt Solution; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NOX, NADPH oxidase; OPZ, opsonized zymosan; PBS, phosphate Buffered Saline; Phox, phagocyte oxidase; PiB, diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[Imn]-phenanthroline-2,7-diacetate; Pin1, prolyl *cis/trans* isomerase; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophils; RA, rheumatoid arthritis; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide.

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The neutrophil enzyme responsible for ROS production is the nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase [11–13], which produces superoxide anion ( $O_2^{\cdot-}$ ), the source of other ROS molecules such as hydrogen peroxide ( $H_2O_2$ ) which is used by myeloperoxidase (MPO) to produce hypochlorous acid (HOCl), all of which cause the destruction and death of pathogens in the phagosome [5–10]. The phagocyte NADPH oxidase is a multi-component enzyme system consisting of six proteins: two transmembrane proteins (gp91phox or NOX2 and p22phox) and four soluble cytosolic proteins (p40phox, p67phox, p47phox and the small G proteins, Rac1/2) [11,12]. In resting cells, NADPH oxidase is in a dormant state with spatial separation of the components. After neutrophil stimulation by various agents such as fMLF or phorbol myristate acetate (PMA), the cytosolic subunits p47phox, p67phox, and p40phox are phosphorylated and translocate to associate with gp91phox and p22phox in the membrane to assemble the active NADPH oxidase [14]. Phosphorylation of p47phox on multiple serines plays an important role in NADPH oxidase activation [14,15]. NADPH oxidase activation can be enhanced or primed in phagocytes by various agents such as TNF $\alpha$ , GM-CSF, IL-8 and TLR agonists such as the bacterial lipopolysaccharide (LPS), CL097 [8,16–20].

Physiological priming of the neutrophil NADPH oxidase is thought to have many beneficial effects, such as efficient bacterial and fungal killing [8]. However, excessive priming of NADPH oxidase could lead to excessive ROS production, which contributes to tissue damage involved in inflammatory diseases and thus may be involved in inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and acute respiratory distress syndrome (ARDS) [8,9,21–25]. RA is a systemic inflammatory disease that most commonly affects the joints [7,9,26]. The pathophysiology of RA involves dysregulated cytokine production and neutrophil accumulation in the synovial fluid [7,9,26]. Pro-inflammatory cytokines such as TNF $\alpha$ , GM-CSF, IL-1, IL-8 and excessive neutrophil activation have been implicated in rheumatoid tissue damage. The pathways involved in excessive neutrophil activation by these cytokines are not fully understood.

The pro-inflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) plays an important pathogenic role in several inflammatory diseases [27,28]. Human GM-CSF is a 14.7 kDa glycosylated protein; it was originally discovered as a hematopoietic cytokine capable of generating granulocyte and macrophage precursors in the bone marrow [27,28]. GM-CSF is produced by T cells, macrophages, fibroblasts, and endothelial cells [27,28]. GM-CSF is also capable of upregulating mature cell functions such as neutrophil ROS production, degranulation and survival [16,29,30]. GM-CSF acts on cells by binding to a specific membrane receptor. The GM-CSF receptor is composed of two chains called  $\alpha$  and  $\beta$  [31]. Binding of GM-CSF to its receptor activates a number of signal transduction pathways in human neutrophils, including the protein tyrosine kinase JAK2, the src tyrosine kinase lyn, STAT and PI3K [32–35]. The molecular mechanisms by which GM-CSF upregulates or primes the respiratory burst in human neutrophils are not understood. GM-CSF is known to induce NOX2 translocation to the membranes and the phosphorylation of p47phox at Ser345 in human neutrophils [16,36,37]. However, the link to NADPH oxidase priming in neutrophils is still not understood.

The peptidyl-prolyl *cis-trans* isomerase (PPIase), Pin1 is an enzyme that catalyzes the isomerization of prolyl peptide bonds from *cis* conformation to *trans* orientation [38,39]. Pin1 recognizes a phospho-Ser/Thr-Pro sequence and has been shown to be a crucial regulator of many proteins phosphorylated on serine/threonine [38,39]. Pin1 has been implicated in a variety of diseases, including cancer, cardiovascular disease and Alzheimer's disease [40,41]. We have shown that Pin1 is involved in the priming of NADPH oxidase induced by TNF $\alpha$ , CL097 (a TLR7/8 agonist) and LPS in human neutrophils [19,20,42]. However, the role of Pin1 in GM-CSF-induced priming of neutrophil ROS production is not known. In this study, we demonstrate that Pin1 mediates the GM-CSF-induced priming of NADPH oxidase in human neutrophils

and in primed neutrophils isolated from synovial fluid of RA patients.

## 2. Materials and methods

### 2.1. Reagents

GM-CSF was from Peprotech. Juglone, PiB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[*lmn*]-phenanthroline-2,7-diacetate), phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), protease and phosphatase inhibitors were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). The Pin1 peptide substrate was purchased from Bachem. Sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and Western blotting reagents were purchased from Bio-Rad (Hercules, CA, USA). The rabbit polyclonal antibodies against phospho-Ser345 of p47phox and anti-p47phox were produced in our laboratory as described elsewhere [16,19]. Anti-Pin1 antibody was obtained from Santa Cruz. HRP-conjugated goat anti-mouse was obtained from Santa Cruz Biotechnology Inc (Heidelberg, Germany).

### 2.2. Ethical statement

After signing informed consent, neutrophils were isolated from venous blood of healthy volunteers and from knee joint synovial fluid of RA patients who met the American College of Rheumatology criteria [26] and who were not receiving anti-cytokine biotherapy. Data collection and analysis were performed anonymously. All experiments were supported by the Institutional Review Board and the ethics Committee of INSERM.

### 2.3. Isolation of human neutrophils from blood and synovial fluid

Neutrophils were isolated from the venous blood of healthy volunteers by polymorphprep gradient centrifugation as described previously [16,43]. After hypotonic lysis of erythrocytes, the neutrophil pellets were collected and washed in PBS before cell counting. Viability was assessed by trypan blue dye exclusion. This isolation method consistently yielded PMN that were 96 % pure and 99 % viable. Synovial fluid was diluted twice in sterile PBS and cells were isolated by one-step polymorphprep gradient centrifugation at 500 g for 30 min at 22 °C. The neutrophil band was collected and the cells were washed in PBS and counted.

### 2.4. Pin 1 activity assay

Pin1 activity was determined as previously described [42,44] with some modifications. Briefly, neutrophils were resuspended in an ice-cold lysis buffer containing 50 mM HEPES pH 7.5, 0.25 % CHAPS, 100 mM NaCl, 1 mM  $\beta$ -glycerophosphate, 5 mM NaF and 1 mM EGTA, at  $10^7$ /ml, and lysed with several 2 ml syringe pressures. The assay mixture contains 369  $\mu$ L HEPES buffer (50 mM HEPES (pH 7.8), 25  $\mu$ L (60 mg/ml) chymotrypsin solution (Sigma-Aldrich), 6  $\mu$ L (6 mM) of the peptide Suc-Ala-Glu-Pro-phe-pNA (BACHEM), and 50  $\mu$ L cell lysate ( $10^6$  cell equivalent). The absorbance change due to the release of *p*-nitroaniline (pNA) was monitored at 410 nm at 4 °C using a spectrophotometer (CARY 3500; Agilent).

### 2.5. Luminol-enhanced chemiluminescence assay

To determine ROS production we used luminol-enhanced chemiluminescence method [45]. Briefly, neutrophils ( $2.5 \times 10^5$ ) were resuspended in 0.5 mL of HBSS containing 10  $\mu$ M of luminol with or without different concentrations of juglone or PiB (50  $\mu$ M), for 20 min at 37 °C, GM-CSF was added for 20 min; then fMLF ( $10^{-7}$  M) was used to stimulate the cells. Chemiluminescence was recorded with a luminometer (LB937; Berthold-Biolumat).

## 2.6. Bacterial expression of Pin1

GST-Pin1 was expressed in *E. coli* (BL21) grown at 37 °C and then induced with 0.1 mM and 0.2 mM isopropylthiogalactoside at 37 °C for 3 h. Cells were harvested by centrifugation (4000 g, 20 min, 4 °C) and the pellet was resuspended in lysis buffer [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 % (v/v) Triton X-100, protease inhibitors]. Cells were lysed by six sonications of 30 s each. After centrifugation (4000 rpm, 20 min, 4 °C), GST-recombinant Pin1 was purified using glutathione-Sepharose 4B beads [42].

## 2.7. GST pull-down assay

One microgram each of GST-Pin1 was incubated with glutathione-Sepharose beads and neutrophil lysates in interaction buffer (PBS; 1 % Chaps; 0.1 mM DTT; 5 mM NaF and 1 mM β-glycerophosphate) for 2 h. After washing, the complex was released by cleavage with thrombin protease overnight at 4 °C and analyzed by SDS-PAGE and Western blot, using specific antibodies [42].

## 2.8. SDS-PAGE and Western blotting

Neutrophils (1x10<sup>7</sup> cells in 500 μl of HBSS) were incubated with or without increasing of GM-CSF concentrations for 20 min at 37 °C. The reaction was stopped by adding 125 μl of concentrated modified sample buffer (5X) containing 50 μg/mL pepstatin, 50 μg/mL leupeptin, 25 mM NaF, 12.5 mM Na<sub>3</sub>VO<sub>4</sub>, 12.5 mM EDTA, 12.5 mM EGTA, 6.25 mM p-NPP and 50 μg/mL aprotinin [46]. Samples were denatured in boiling water (100 °C) for 3 min and stored at -80 °C until use. Samples were thawed and sonicated for 10 s before use and then they were subjected to 10 % SDS-PAGE using classical techniques [46]. Separated proteins were transferred to nitrocellulose membranes [43]. Nitrocellulose membranes were blocked with 5 % nonfat dry milk in a mixture of tris-buffered saline and tween-20. The membranes were incubated overnight at 4 °C in solution containing specific relevant primary antibodies; anti-phospho-S345 (1:10000) and p47phox(1:5000) or anti-Pin1 (1/1000), followed by incubation with secondary antibodies (Santa Cruz, Heidelberg, Germany). Blots were visualized by using ECL Western blotting reagents (Amersham Pharmacia).

## 2.9. Statistical analysis

The experiments were repeated at least three times. All results are expressed as mean ± SEM. Significant differences (p < 0.05) were

evaluated by Student's *t*-tests and one-way ANOVA followed by Tukey's post-hoc test using Prism 8.0 software (GraphPad).

## 3. Results

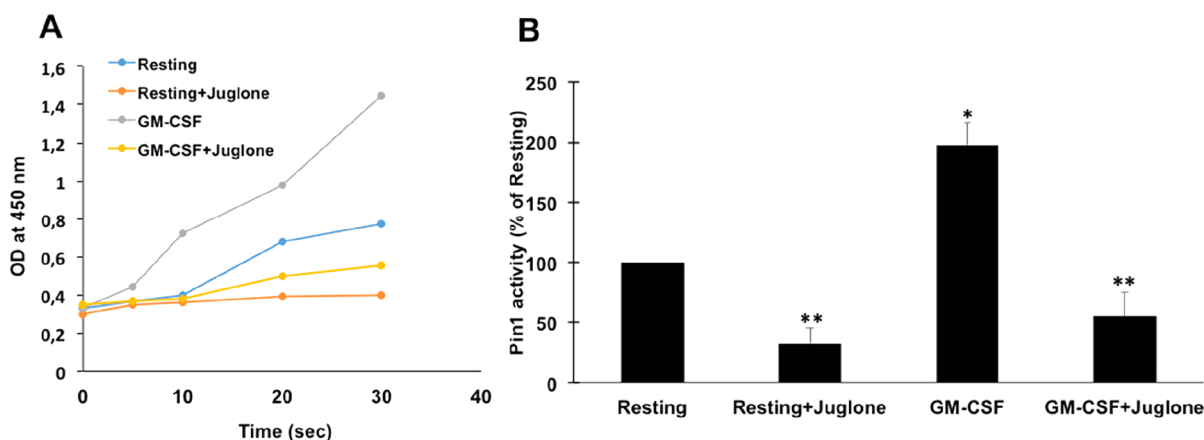
### 3.1. GM-CSF induces Pin1 activation in human neutrophils

To investigate the involvement of Pin1 in the priming effect of GM-CSF on neutrophil ROS production, we first wanted to know if GM-CSF was able to stimulate Pin1 activity in human neutrophils. Neutrophils were incubated with GM-CSF for 20 min, in the absence and presence of juglone, a selective inhibitor of Pin1 activity [47], and then lysed. Pin1 activity was determined by measuring the absorbance of free pNA resulting from the cleavage of Suc-Ala-Glu-Pro-Phe-pNA after it undergoes *cis to trans* conformational changes. The results show that Pin1 is constitutively active in resting neutrophils, as previously observed [42,44], and that GM-CSF strongly increased Pin1 activity (Fig. 1). Treatment of cells with juglone prior to GM-CSF treatment significantly reduced GM-CSF-induced Pin1 activity, demonstrating that this assay is specific for Pin1.

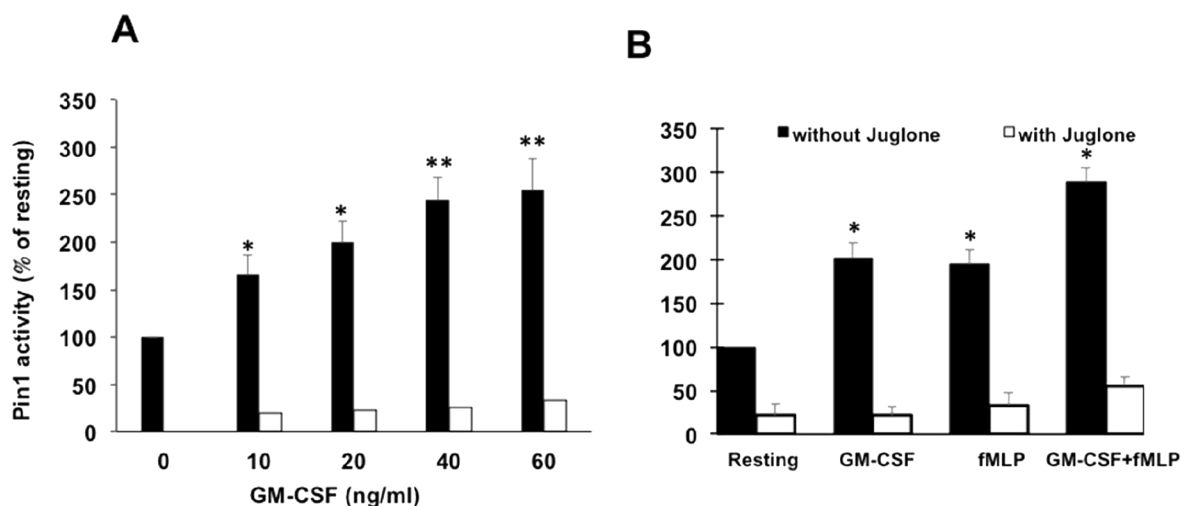
The activation effect of GM-CSF on Pin1 was dose-dependent (Fig. 2A) and very similar to that of fMLF, and both agents further enhanced Pin1 activation (Fig. 2B). The results obtained here show that GM-CSF is able to induce Pin1 activation in human neutrophils and suggest a possible role of Pin1 in GM-CSF-induced neutrophil functions.

### 3.2. Pin1 enzymatic activity is required for GM-CSF-induced priming of ROS production in human neutrophils

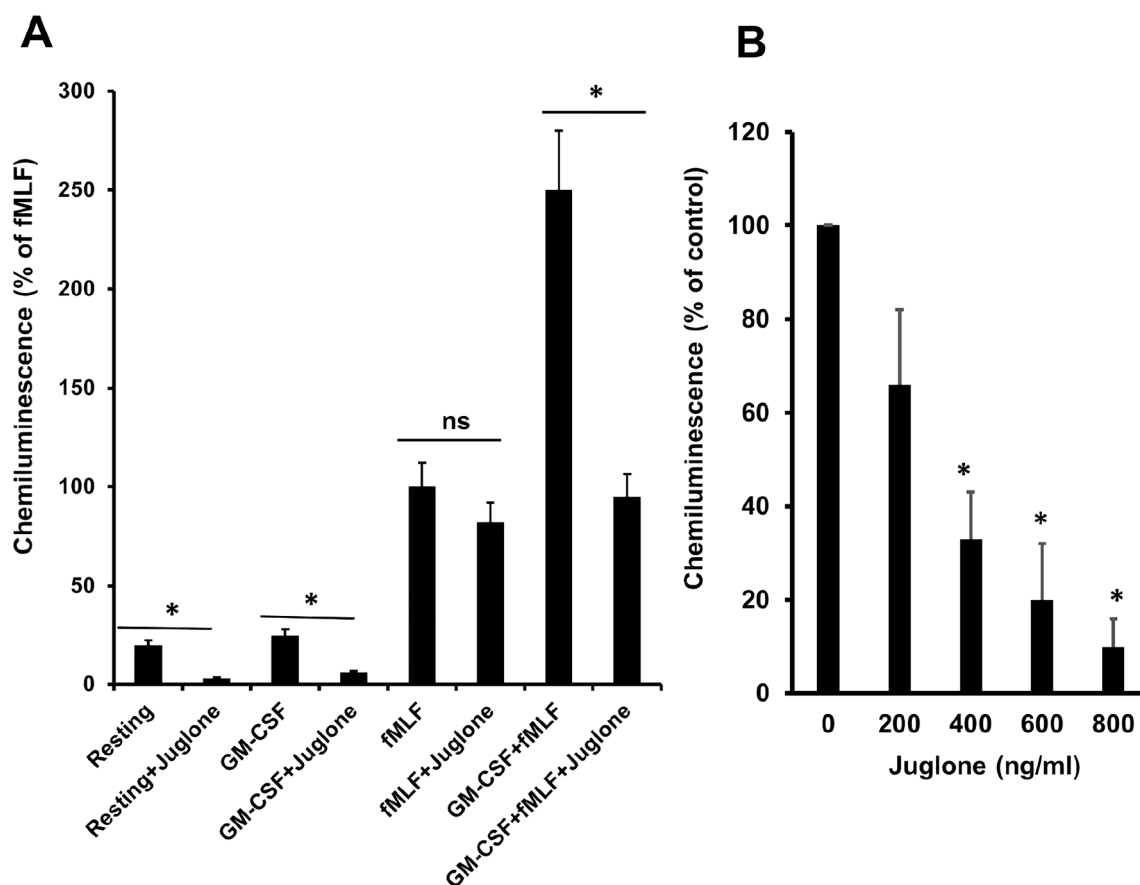
We next investigated the role of Pin1 in GM-CSF-induced priming of ROS production using the selective Pin1 inhibitor juglone. Neutrophils were incubated with juglone for 20 min, treated with GM-CSF for another 20 min, then stimulated with fMLF (10<sup>-7</sup>μM). ROS production was measured using luminol-enhanced chemiluminescence assay. The results show that, as expected GM-CSF alone had no effect on ROS production, but it dramatically enhanced fMLF-induced ROS production and juglone was able to inhibit this priming effect of GM-CSF (Fig. 3A). The juglone inhibitory effect was dose-dependent and ranged from 200 to 800 nM (Fig. 3B). To confirm the involvement of Pin1 in this process, we used PiB, another inhibitor that is chemically unrelated to juglone and has been described to be more specific [48]. These results show that PiB inhibited GM-CSF-induced priming of neutrophil ROS production (Supplementary Figure 1). Juglone and PiB exert their inhibitory effects without affecting cell viability even at the highest concentration used



**Fig. 1.** GM-CSF induced Pin1 activation in human neutrophils and juglone significantly decreased Pin 1 activity. (A) Neutrophils were incubated in the absence and presence of juglone (400 nM) for 20 min and then treated with GM-CSF (20 ng/mL), during 20 min before lysis. Pin1 activity was determined by measuring the absorbance of free pNA cleaved from Trp-Phe-Tyr-Ser (PO<sub>3</sub>H<sub>2</sub>)-Pro-Arg-pNA Suc. (B) Data from multiple experiments were analyzed using the values of Pin1 activity in resting cells as 100 % and presented as mean plus or minus SEM (n = 4 experiments, \*p < 0.01).



**Fig. 2.** Concentration-dependent effect of GM-CSF-induced Pin1 activation in human neutrophils and comparison with fMLF. (A) Neutrophils were incubated in the presence of different concentrations of GM-CSF (20–60 ng/mL), for 20 min before lysis. Pin1 activity was determined by measuring the absorbance of free pNA cleaved from Trp-Phe-Tyr-Ser (PO<sub>3</sub>H<sub>2</sub>)-Pro-Arg-pNA Suc. Data from several experiments were analyzed using the values of Pin1 in resting cells as 100 % and presented as mean plus or minus SEM (n = 4 experiments, \*p < 0.01). (B) Neutrophils were incubated in the presence of GM-CSF (20 ng/mL), during 20 min or fMLF (10<sup>-7</sup> M) for 1 min or GM-CSF + fMLF before lysis. Pin1 activity was determined by measuring the absorbance of free pNA cleaved from Trp-Phe-Tyr-Ser (PO<sub>3</sub>H<sub>2</sub>)-Pro-Arg-pNA Suc. Data from several experiments were analyzed using the values of Pin1 activity in resting cells as 100 % and presented as mean plus or minus SEM (n = 4 experiments, \*p < 0.01).



**Fig. 3.** Effect of juglone, a Pin1 inhibitor on GM-CSF-induced priming of ROS production by human neutrophils. (A) Human neutrophils ( $0.5 \times 10^6$ /ml) were incubated in the absence or presence of juglone (400 nM) for 20 min at 37 °C, treated with GM-CSF and then stimulated with fMLF (10<sup>-7</sup> M). ROS production was measured using a luminol-amplified chemiluminescence technique. Total chemiluminescence in each experimental condition is expressed as mean plus or minus SEM of 3 experiments. \*p < 0.01 as compared to fMLF alone (control 100 %). (B) Human neutrophils ( $0.5 \times 10^6$ /ml) were incubated in the absence or presence of increasing concentrations of juglone for 20 min at 37 °C, then GM-CSF (20 ng/mL) was added for 20 min before stimulation with fMLF (10<sup>-7</sup> M). ROS production was measured using a luminol-amplified chemiluminescence technique. Total chemiluminescence in each experimental condition is expressed as the mean plus or minus SEM of 3 experiments. p < 0.001 as compared with control in the absence of juglone (100 %), \*p < 0.01.

(Supplementary Figure 2). Obtaining the same result with two different inhibitors, strongly supports the role of Pin1 in GM-CSF-induced priming of ROS production in human neutrophils.

### 3.3. GM-CSF induces phosphorylation of p47phox at serine 345 and interaction with Pin1

Priming of the NADPH oxidase in neutrophils is accompanied by several processes including the phosphorylation of p47phox at Ser345, the binding site of Pin1 [8,16,42]. To further understand the mechanisms of GM-CSF-induced priming of ROS production by neutrophils, we examined the interaction of Pin1 with phosphorylated p47phox (Ser345). As expected, the results show that treatment of neutrophils with GM-CSF induced a significant phosphorylation of Ser345 of p47phox (Fig. 4A) and interestingly GM-CSF induced the binding of p47phox to Pin1 (Fig. 4B). TNF $\alpha$  is known to induce these processes and was used as a positive control. These results clearly demonstrate that GM-CSF induces Pin1-p47phox interaction.

### 3.4. Pin1 activity is increased in neutrophils isolated from synovial fluid of RA patients and ROS hyperproduction by these neutrophils is inhibited by Pin1 inhibitors

Given the deleterious role of GM-CSF in rheumatoid arthritis (RA) and the involvement of Pin1 in GM-CSF- but also in TNF $\alpha$ -induced priming of neutrophil ROS production, we investigated Pin1 activity and its role in ROS hyperproduction by neutrophils from RA patients. We and others have previously shown that neutrophils isolated from synovial fluid of RA patients produce more ROS than blood neutrophils, both under basal conditions and when stimulated with fMLF [16,23,49]. Furthermore, phosphorylation of p47phox (on Ser345), ERK1/2 and p38MAPK is increased in synovial neutrophils from RA patients compared to blood neutrophils [16]. Here we found that Pin1 activity was higher in neutrophils isolated from synovial fluid of RA patients compared to resting neutrophils isolated from blood of healthy donors (Fig. 5).

In resting neutrophils from synovial fluid, the magnitude of the increase in Pin1 activity was similar to that observed in circulating

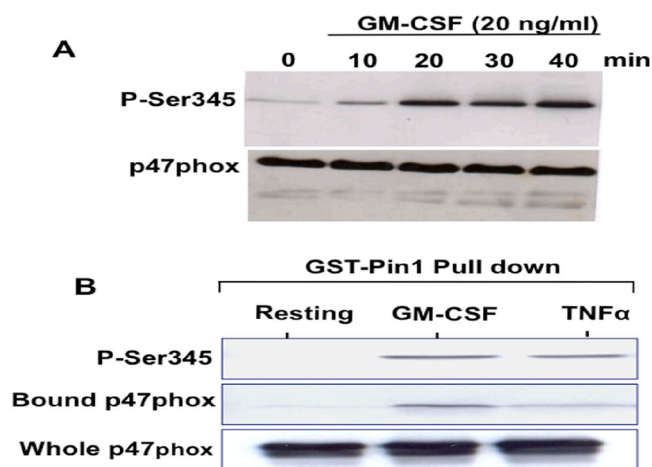
neutrophils treated with TNF $\alpha$  *in vitro*. Significantly, the Pin1 inhibitors juglone and a Pin1-peptide inhibitor (PPin) strongly inhibited basal (Fig. 6A and 6C) and fMLF-induced ROS production by neutrophils from synovial fluid of RA patients (Fig. 6B and D). Thus, Pin1 activity is elevated in neutrophils isolated from synovial fluid of RA patients, and Pin1 inhibitors strongly inhibit ROS hyperproduction by these cells. These findings suggest that Pin1 may mediate the priming of synovial neutrophils in RA, and that Pin1 inhibitors may have anti-inflammatory properties.

## 4. Discussion

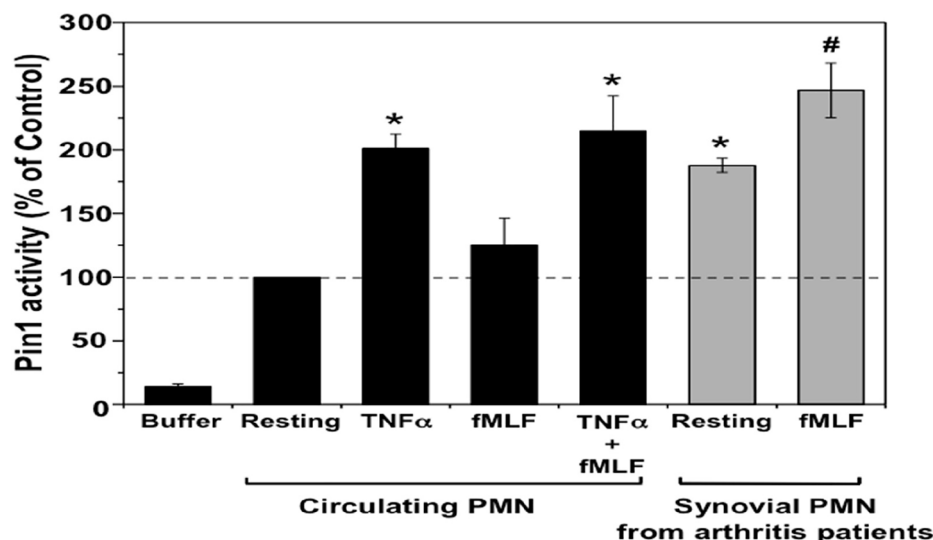
GM-CSF is a pro-inflammatory cytokine capable of upregulating several neutrophil functions, including superoxide anions production by the phagocyte NADPH oxidase, degranulation and lifespan. GM-CSF-induced priming of superoxide production has been known for several years, but the pathways involved in this process are not well characterized. Here we show that the peptidylprolyl *cis/trans* isomerase Pin1 controls the GM-CSF-induced NADPH oxidase priming in human neutrophils and neutrophils isolated from synovial fluid of patients with rheumatoid arthritis, which are known to be primed *in vivo*. We showed a concomitant increase in Pin1 activity and ROS generation, while both juglone and a peptide inhibitor effectively inhibited ROS hyperproduction by the same cells. Thus, Pin1-catalyzed prolyl isomerization is a novel mechanism regulating GM-CSF-induced neutrophil NADPH oxidase priming and subsequent ROS hyperproduction by neutrophils at inflammatory sites.

To investigate the role of Pin1 in GM-CSF treated neutrophils, we first examined the effect of GM-CSF on Pin1 enzymatic activity in neutrophils. As we previously found, Pin1 is constitutively active in neutrophil lysates [42,44], and interestingly GM-CSF is able to increase Pin1 activation in neutrophils. GM-CSF was able to increase Pin1 activation in a manner comparable to fMLF. GM-CSF has also been shown to increase Pin1 activation in human eosinophils [50]. This leads to an interaction of Pin1 with phosphorylated Bax and GM-CSF-induced survival. However, the pathways involved in GM-CSF-induced Pin1 activation in neutrophils and eosinophils are unknown and require further studies.

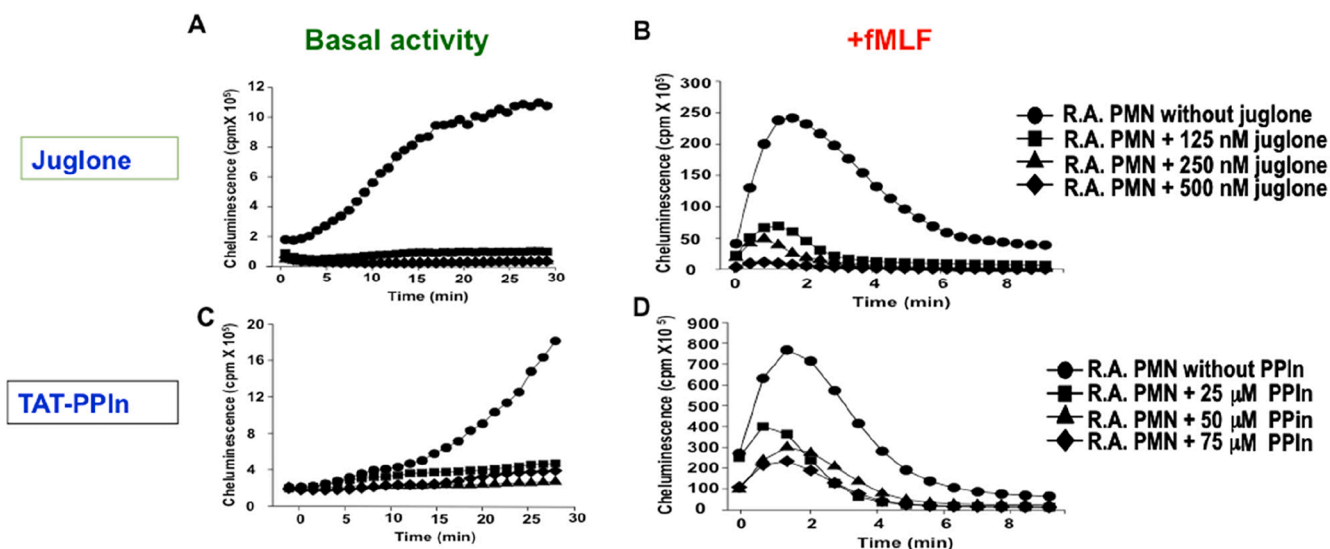
The GM-CSF receptor consists of two chains/subunits: a low affinity alpha subunit (CD116) which is specific for GM-CSF, and a high affinity beta chain (CD131) shared with IL-3 and IL-5 [31]. GM-CSF binding to its receptor activates a number of signal transduction pathways in human neutrophils, including the protein tyrosine kinases (PTKs) JAK2 and the src-tyrosine kinase lyn, which transduce the GM-CSF signal by phosphorylating other proteins such as STAT, PKB and PI3K and the MAPK family ERK1/2. JAK2 is the only member of the JAK family that is activated by GM-CSF in neutrophils and it is directly or indirectly involved in the tyrosine phosphorylation of p85, the regulatory subunit of PI3K. We have previously shown that wortmannin, a PI3Kinase inhibitor, PD98059, an ERK1/2 inhibitor, and AG490, a JAK2 inhibitor inhibit GM-CSF-induced p47phox phosphorylation at Ser345 and priming of NADPH oxidase [37]. Phospho-Ser345 is located in a proline-rich region (-PX-phosphoSP-) that can exist in the *cis* or *trans* conformation, and proline isomerization has a regulatory effect involving subtle conformational changes. Prolyl *cis/trans* isomerization is a spontaneous process but can also be catalyzed by several enzymes. Pin1 is a unique prolyl isomerase that specifically recognizes phosphorylated serine or threonine immediately adjacent to the a proline and then isomerizes the peptide bond. In contrast to the other peptidyl-prolyl isomerases, activated Pin1 binds only to the phosphorylated (Ser/Thr)-Pro sequence [38,51]. The NADPH oxidase component p47phox is phosphorylated on Ser345-Pro which is a Pin1 recognition motif [16,42]. We have previously shown that TNF $\alpha$ , another pro-inflammatory cytokine induced Pin1 activation and its binding to phosphoSer345 of p47phox. Therefore, we investigated the effect of GM-CSF on p47phox phosphorylation and its binding to Pin1. Interestingly,



**Fig. 4.** GM-CSF induced phosphorylation of p47phox on Ser345 and its interaction with Pin1. (A) Neutrophils ( $10 \times 10^6$  cells/mL) were incubated with or without GM-CSF for 20 min at 37 °C. Cells were lysed and proteins were denatured and analyzed by SDS-PAGE and Western blot using an anti-phospho-Ser345-p47phox and an anti-p47phox antibodies. Experiments were repeated 3 times. (B) Neutrophils ( $10 \times 10^6$  cells/mL) were incubated with or without GM-CSF or TNF for 20 min at 37 °C. Cells were lysed and incubated with GST-Pin1, washed, denatured and analyzed by SDS-PAGE and Western blot using anti-p47phox and anti-phospho-Ser345-p47phox. Experiments were repeated 3 times.



**Fig. 5.** Pin1 activity is upregulated in neutrophils isolated from synovial fluid of rheumatoid arthritis patients. Neutrophils (PMN) isolated from circulating blood of healthy donors and synovial fluid of RA patients were subjected to different TNF $\alpha$  and fMLF treatments as indicated in the figure, lysed and Pin1 activity was measured by measuring the absorbance (390 nm, 4 min) of *p*-nitroaniline cleaved from the peptide Trp-Phe-Tyr-Ser(PO3H2)-Pro-Arg-pNA (\*  $p < 0.01$  compared to resting circulating PMN; #  $p < 0.01$  compared to fMLF-stimulated circulating PMN).



**Fig. 6.** Pin1 inhibitors prevent neutrophil ROS hyperproduction in neutrophils isolated from synovial fluid of rheumatoid arthritis patients. Neutrophils isolated from synovial fluid of rheumatoid arthritis patients ( $5 \times 10^5$  cells) were incubated with juglone (A and B) or Pin1-peptide inhibitor (PPIin) (C and D) for 30 min, then ROS production was measured by a luminol-amplified chemiluminescence technique in the presence or absence of fMLF ( $10^{-7}$  M). Data are representative of 10 independent experiments with cells from 10 different patients.

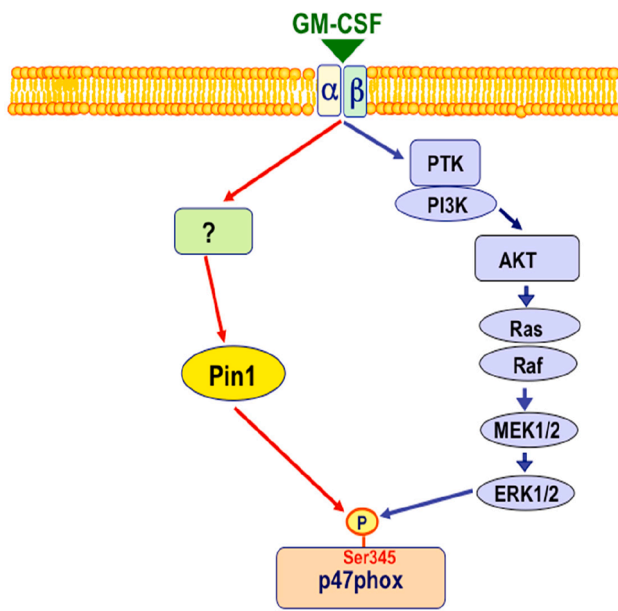
GM-CSF mainly induced the phosphorylation of p47phox on Ser345 and the interaction of Pin1 and p47phox. Taken together, these data suggest that the “JAK2-PI3Kinase-ras-Raf-MEK-ERK1/2-p47phoxSer345-Pin1” axis controls GM-CSF-induced neutrophil priming (Fig. 7).

Notably, the Pin1 inhibitor, juglone at low concentrations specifically inhibited the priming effect of GM-CSF but did not alter superoxide production induced by fMLF alone, suggesting that Pin1 inhibitors may serve as anti-inflammatory agents while preserving the physiological bactericidal function of neutrophils. Juglone may also affect other enzymes, that are directly or indirectly involved in the regulation of the NADPH oxidase. Because neutrophils are terminally differentiated and short-lived cells, they are resistant to transfection. An alternative strategy to confirm the involvement of Pin1 is to use another inhibitor chemically unrelated to juglone. Indeed, a more selective Pin1 inhibitor has been described, it is called PiB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-

tetraoxobenzo[lmn]-phenanthroline-2,7-diacetate) [48]. The results show that PiB inhibited GM-CSF-induced priming of neutrophil ROS production. The fact that the same result was obtained with both inhibitors, strongly supports the role of Pin1 in the GM-CSF-induced effect.

We have previously shown that Pin1 is involved in TNF $\alpha$ , CLO97 (a TLR7/8 agonist), and LPS-induced priming of NADPH oxidase in human neutrophils [19,20,42]. Pin1 may be a common mediator of the priming of NADPH oxidase in human neutrophils.

Pin1 plays a key role in aging, Alzheimer's disease and cancer [40,41,52]. Pin1 also regulates the stability of TGF and GM-CSF mRNA in eosinophils and T lymphocytes [50,53]. In this study, we identify a key role of Pin1 in controlling the degree of NADPH oxidase activation in GM-CSF-primed neutrophils *in vitro*. Similarly, we observed a role for Pin1 in the priming of NADPH oxidase in neutrophils isolated from synovial fluid of RA patients. ROS production by the phagocyte NADPH



**Fig. 7.** Schematic representation of the effect of the pathways involved in GM-CSF-induced priming of NADPH oxidase in human neutrophils. Binding of GM-CSF to its receptor induces the activation of JAK2 and Lyn tyrosine kinases which activate PI3K-AKT-Raf1-MEK1/2 and ERK1/2 to phosphorylate p47phox on Ser345. GM-CSF also induced the activation of the prolyl-peptidyl isomerase Pin1, which binds to phosphorylated Ser345 to induce a conformational change of p47phox. These pathways are activated in synovial neutrophils from RA patients.

oxidase complex plays an important role in host defense, but excessive ROS production can damage healthy bystander tissues, thereby contributing to inflammatory diseases such as RA. The pathophysiology of RA involves dysregulated cytokine production and neutrophil accumulation in the synovial fluid [6–8]. We have previously reported increased NADPH oxidase activity and excessive ROS generation through increased p47phox(Ser345) phosphorylation in neutrophils isolated from synovial fluid of RA patients, as compared to those isolated from blood [16]. Here, we observed increased Pin1 activity in neutrophils isolated from RA patients, and a strong decrease in ROS hyperproduction after treatment with Pin1 inhibitors. These results suggest a regulatory role of Pin1 in NADPH oxidase activity and ROS production in an inflammatory environment *in vivo*. High levels of proinflammatory cytokines are present in the synovial fluid of RA patients. These include TNF $\alpha$  and GM-CSF, which activate ERK1/2 or p38MAPK, induce Ser345 p47phox phosphorylation, and prime NADPH oxidase activity in human neutrophils. It was also shown that Pin1 expression was increased in human and mouse synovial tissues and that juglone was able to alleviate RA progression in mice [54,55]. Furthermore, Pin1 may play a key role in the development of systemic lupus erythematosus [56]. Our results obtained on neutrophils and all the other results on other tissues point out to the importance of Pin1 in inflammation.

In conclusion, the high levels of pro-inflammatory cytokines in the synovial fluid of RA patients, strongly supports a central role of these cytokines in the pathogenesis of the associated joint damage. In this study, we show that excessive neutrophil ROS production induced by GM-CSF and at site of inflammation is controlled by Pin1. Inhibition of Pin1 may be a novel strategy to treat inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and inflammatory lung disease.

#### Institution Review Board statement

The collection and analyses of data were performed anonymously. All experiments were supported by the Inserm Institutional Review

Board and ethics committee.

#### Author contributions

TB and HR designed and performed the experiments and analyzed the data. SB performed experiments. RAD performed the experiments. GH, MAGP, PMCD and JEB designed the experiments and analyzed the data. All authors contributed to writing the manuscript.

#### CRediT authorship contribution statement

**Tarek Boussetta:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Houssam Raad:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Samia Bedouhene:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Riad Arabi Derkawi:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marie-Anne Gougerot-Pocidallo:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Gilles Hayem:** Writing – review & editing, Visualization, Validation, Conceptualization. **Pham My-Chan Dang:** Writing – review & editing, Visualization, Validation, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Jamel El-Benna:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

No data was used for the research described in the article.

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#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2024.112425>.

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