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Inhibition of NET formation by polydatin protects against collagen-induced arthritis

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a systematic, inflammatory, autoimmune disease, associated with a high number of disabilities. Increasing evidence has demonstrated that neutrophil extracellular trap (NET) formation plays a significant role in the pathogenesis and progression of RA. In this study, we have aimed to investigate the effects of polydatin (PD) on NET formation and its effects on disease activity in a collagen-induced arthritis (CIA) mouse model.

Methods: In the presence of PD or vehicle, neutrophils isolated from RA patients and mice were treated with phorbol 12-myristate 13-acetate (PMA) for 4 h, and NET formation investigated. For in vivo experiments, PD was administered intraperitoneally (45 mg/kg per day) to collagen-induced arthritis (CIA) mice. The incidence and severity of collagen-induced arthritis were assessed and NET deposition tested.

Results: In vitro, PD significantly suppressed NET formation of neutrophils from RA patients. Consistently, decreased NETs were observed in PD treated bone marrow-derived neutrophils. In CIA mouse model, PD treatment delayed the onset of arthritis and attenuated arthritis severity. Compared with vehicle-treated CIA mice, the deposition of NETs in ankle joints was also reduced in PD-treated CIA mice.

Conclusion: In this study, we found that PD treatment markedly inhibited NET formation and protected CIA mice from the development of arthritis. These findings suggest that inhibition of NET formation by PD may serve as a novel mechanism for the treatment of RA.

1. Introduction

Rheumatoid arthritis is one of commonly chronic, systematic, autoimmune diseases manifested by joint synovial chronic inflammation, pannus formation, articular cartilage destruction, and autoantibody production, mainly anti-citrullinated protein antibodies (ACPAs) [1,2]. The prevalence of rheumatoid arthritis is approximately 0.5–0.8% in the general population, but its physical disability rate is up to 79.4% [3], increasing the burden of healthcare systems. A deeper understanding of the underlying mechanisms of RA pathogenesis contributes to uncovering a new potential approach for treatment. In addition to the central role of the adaptive immune system in RA pathogenesis, locally resident cells, such as neutrophils and macrophages mainly contribute to inflammation responses and tissue damage [4]. As the most abundant cells in synovial fluid, neutrophils have been implicated in RA progression [5]. NETosis was first reported as an important step in killing bacteria by neutrophils in 2004 [6]. During NETosis, decondensed chromatins decorated with intracellular components, such as neutrophil elastase (NE), myeloperoxidase (MPO) and proteinase3 (PR3) are extruded into extracellular space, forming web-like structures, neutrophil extracellular traps (NETs) [7]. Emerging evidence has indicated a central role of NET formation in RA pathogenesis and progression. Citrullinated antigens targeted by autoantibodies to citrullinated protein antigens (ACPAs) were reported mostly derived from NETs. A previous study had reported that antibodies against citrullinated histone H4 in NETs were tested in sera from more than 60% of RA patients [8]. In addition, in RA about 40% of monoclonal antibodies produced by B cells in synovial tissue showed a higher reactivity

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against citrullinated histones H2A/H2B, citrullinated fibrinogen, and citrullinated vimentin [9]. Furthermore, RA NETs induced the activation and inflammatory cytokine release of fibroblast-like synoviocytes (FLS), causing damaged joints [10]. Autoantibodies and inflammatory cytokines can also stimulate neutrophils to undergo NETosis, producing a vicious cycle of cytokine release and autoantibody formation [10].

PD, a monomer extracted from the roots of Polygonum Cuspidatum, has gained great attention for its numerous biological functions, especially anti-oxidation and anti-inflammation effects [11]. Many studies have reported that PD could significantly inhibit reactive oxygen species (ROS) overproduction [12–14]. Indeed, ROS overproduction is necessary for NET formation [15]. However, its effects on NET formation in RA and clinical effects on RA have not yet been investigated. Therefore, our present study aimed to evaluate the modulatory effects of PD on collagen-induced arthritis (CIA) and explore potential mechanisms by which PD could exhibit beneficial effects on RA.

2. Methods and materials

2.1. Animals

Six to eight-week-old male DBA/1 mice, purchased from Shanghai Slac Laboratory Animals (Shanghai, China), were bred and housed under specific pathogen-free (SPF) conditions in the Laboratory Animal Center of Southern Medical University (Guangzhou, China). The animal experiments were carried out in accordance with the animal experimental ethical guidelines of Southern Medical University (No. L2017123).

2.2. Sample collection and neutrophil isolation

Peripheral blood samples from RA patients, who were diagnosed by clinicians according to the 2010 American College of Rheumatology/EULAR RA classification criteria in the Third Affiliated Hospital of Southern Medical University, were collected. Neutrophils from these RA patients were isolated by dextran sedimentation and centrifugation, as previously described [16]. For isolation of mouse bone marrow-derived neutrophils (BMDNs), tibiales, and femurs were obtained after euthanasia and then bone marrow cells were collected. According to the instructions of the manufacturer, mice BMDNs were isolated by using a neutrophil cell isolation kit (TBD Sciences, Tianjin, China).

2.3. Cell viability assay

According to the instructions of the manufacturer, the effect of PD (a generous gift from Professor Ke-seng Zhao, Guangdong Key Laboratory of Shock and Microcirculation Research of Southern Medical University) on human neutrophil viability was assessed by using Cell Couting Kit (CCK-8) (Dojindo, Beijing, China). Neutrophils were incubated in the presence of various concentrations of PD (0, 50, 75, 100 and 125ug/mL) and finally the optical density (OD) value was tested at 450 nm on a microplate reader.

2.4. Quantification of NETs

To determine the effects of PD on NET formation, neutrophils isolated from RA patients and mice bone marrow (3×10^5 cells/well in 200 μl) were seeded into black, flat-bottomed, 96-well plates and pretreated without PD or with PD (100 μg/ml) for 1 h. In experiments with 25 nM PMA (Sigma-Aldrich, St. Louis, USA) for human neutrophils or 50 nM PMA for mice BMDNs, the cells were incubated at 37 °C with CO_2 (5%) for 4 h. After incubation, the extracellular DNA was stained with dye-SYTOX green (Thermo Fisher Scientific, USA), which could not bind to intracellular DNA if the cell membrane was intact. Fluorescent intensity was detected using Spectra Max M3 fluorescent plate reader at excitation 485 nm and emission 520 nm.

2.5. Immunofluorescent staining of NET formation

With or without pretreatment of PD, 1.5×10^5 neutrophils from RA patients and mice were directly placed onto poly-L-lysine-coated coverslips and then separately stimulated with 25 nM PMA and 50 nM PMA for another 4 h. For staining preparation, neutrophils were fixed with 4% paraformaldehyde and blocked with 5% fetal bovine serum (FBS). Finally, the coverslips were stained with rabbit anti-MPO antibody (Abcam, catalog ab208670, UK) for MPO, followed by incubation with a Cy3-conjugated goat secondary antibody against rabbit IgG (Servicebio, catalog GB21303, China). For deoxyribonucleic acid (DNA) visualization, it was stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, USA). Images were collected with a fluorescence microscope, typically at original ×400 magnification.

To observe NET formation more directly, SYTOX green was added in the cells and the unfixed NETs were directly imaged with a fluorescence microscope as described previously [17].

2.6. Induction of collagen-induced arthritis mouse model

Collagen-induced arthritis was induced in DBA/1J mice as previously described [18]. For preparation of collagen type II (CII) emulsion, 2 mg/ml of bovine type II collagen (Chondrex Inc., Redmond, USA) was dissolved in 0.05 M acetic acid and then emulsified with an equal amount of Freund's complete adjuvant (Chondrex Inc., Redmond, USA) containing 1 mg/ml M. tuberculosis. Then, CIA mice were immunized twice with the prepared collagen type II (CII) emulsion. On day 0, DBA/1J mice were injected intradermally at the base of their tails (first immunization) with 0.1 ml emulsion. The second immunization was conducted near the primary injection site on day 21 with an injection of 0.1 ml emulsion. The DBA/1 mice were randomly divided into three groups, with 5 mice in each group: control group, vehicle-treated CIA model group and PD-treated CIA model group. In PD-treated group, DBA/1J mice were treated with PD (45 mg/kg per day) intraperitoneally from day 21 to day 45. In control group and vehicle-treated CIA model group, mice received the same vehicle volume (dehydrated alcohol-propylene glycol-Na2CO3-NaHCO3 buffer (pH 8.5)) every day until the end of the experiment.

2.7. Arthritis assessment and histological analysis

Arthritis incidence and their severity were evaluated in mice by two independent observers. The severity of arthritis was scored using a visual assessment scoring system from Day 21. The scoring system was shown in detail as follows: 0, normal, no redness or swelling in joints; (1) mild, the ankle or wrist became slightly red and swollen, or apparent redness and swelling occurred in the individual's digits; (2) moderate, red and swelling were observed in the ankle or wrist; (3) serious redness and swelling occurred in the entire paw; (4) severe redness and swelling encompassed multiple joints. The cumulative value of four paws was calculated as the total clinical arthritis score per mouse, with a maximum score of 16 for each mouse.

On day 45, after the first immunization, all mice were sacrificed and joints were removed for further analysis. For histological analysis, mice joints were fixed with 10% formalin and decalcified with 5% formic acid. Then the samples were embedded in paraffin and cut into 4 μm sections in a routine manner. For assessment of joint histological changes, sections were stained with hematoxylin and eosin (H&E).

2.8. Measurement of serum autoantibodies

The serum levels of anti-collagen II IgG2a antibody were measured by Mouse Anti-Bovine Type II Collagen IgG2a Ab Subtype Assay Kit (Chondrex), following the manufacturer's instructions.

2.9. Assessment of NET formation in mouse ankle joints

To assess NET formation in ankle joints, the formalin-fixed and paraffin-embedded joint sections were prepared. After blocking with 5% FBS, the sections were incubated with the primary antibody against MPO overnight at 4 °C. After washing with PBS three times, they were stained with Cy3-conjugated goat anti-rabbit IgG antibody for 1 h. DAPI (Thermo Fisher Scientific, USA) was used to stain these sections of chromatin. Here, the chromatin which also stained for MPO positively was recognized as NETs.

2.10. Statistical analysis

All statistical analyses were performed by SPSS 20.0 software and the figures drawn were by GraphPad Prism 6.0. The data of statistical analysis were presented as mean ± standard deviation (SD) or mean ± standard error of mean (SEM). The data of arthritis score were analyzed, using the nonparametric Wilcoxon rank-sum test. One-way analysis of variance (ANOVA) was used to analyze remaining data. A value of P less than 0.05 was considered statistically significant.

3. Results

3.1. PD significantly blocked NET release mediated by RA patient neutrophils

At first, the cytotoxicity of PD on human neutrophils was tested with a CCK-8 assay. As shown in Fig. 1A, PD exhibited no cellular toxicity even at a concentration as high as 125 μg/ml. Here, PD 100 μg/mL was selected for the following vitro experiment.

PD has been reported to exhibit strong anti-oxidant and anti-inflammatory properties. Evidence has identified the important role of ROS overproduction in NET formation. However, it was not clear whether PMA-induced NET production from RA patient neutrophils could be inhibited by PD treatment. Thus, to determine the effects of PD on NET release, neutrophils isolated from RA patients were stimulated with PMA for NET release in the presence or absence of PD. As expected, PD treatment markedly suppressed PMA-induced NET formation in RA patients (Fig. 1B, C and D).

3.2. PD treatment effectively protected against arthritis in CIA mice.

Many studies have suggested that NET formation plays a vital role in RA pathogenesis and development. Consequently, there is huge interest in whether PD treatment would protect against arthritis in a well-established model of human RA-CIA. The collagen-induced arthritis in mice developed many key manifestations similar to RA in humans; for example, synovitis, cartilage erosion, and MHC II molecular-linked susceptibility; therefore, the CIA model is commonly used in researches related to RA cause and treatment [19,20]. Indeed, in vehicle-treated CIA mice, clinical signs of arthritis started to develop on day 26, after the first immunization, and the incidence of arthritis reached 100% on day 30. Importantly, PD treatment resulted in a delayed onset of CIA and a dramatic reduction in arthritis score (Fig. 2A and B). (Similarly, 60 mg/kg PD showed a protective effect of PD on CIA mice, as shown in Supplementary Fig. 1. The arthritis score was slightly decreased in 30 mg/kg PD-treated mice, but this difference was not statistically significant.) In addition, histological examinations of ankle joints also revealed that PD administration markedly alleviated the severity of arthritis in CIA mice (Fig. 2D). Furthermore, we tested the levels of anti-collagen II IgG2a antibody in serum and found that the serum levels of anti-collagen II IgG2a antibody were obviously reduced in PD-treated CIA mice, compared with vehicle-treated CIA mice (Fig. 2C). Collectively, these results indicated successful establishment of collagen-induced arthritis model and a protective effect of PD on CIA mice.

3.3. PD administration in CIA mice decreased NET formation in inflamed joints

Our data previously demonstrated that PD administration resulted in a significant reduction of NET formation mediated by RA neutrophils in vitro. Next, we further examined the inhibitory role of PD on NET generation in a mouse CIA model. As Fig. 3 shows, abundant NETs were easily detected in joints of vehicle-treated CIA mice. However, PD administration could markedly decrease the NET deposition in joints.

3.4. PMA-induced NET formation was also decreased in PD treated mouse bone marrow-derived neutrophils

As shown above, PD treatment significantly reduced NET deposition in inflamed joints of CIA mice. Therefore, it would be interesting to determine whether PD could suppress NET formation by BMDNs in mice. Consistent with the results observed in RA patients, PD treatment dramatically diminished PMA-induced NET production in BMDNs from mice (see Fig. 4).

4. Discussion

In our study, the beneficial effects of PD on arthritis in a mouse CIA model were demonstrated. PD treatment not only delayed the onset of CIA, but also reduced arthritis score, indicating the anti-arthritis effect. The anti-arthritis effect was further confirmed by significant improvements in the histological features of joints. In line with these findings, Li et al. also found that polydatin effectively ameliorated arthritis symptoms in CIA mice [21]. Interestingly, Kamel also ascertained that PD significantly attenuated articular damage related to complete freund's adjuvant (CFA)-induced arthritis [22]. All these findings emphasized the strong anti-arthritis effects of PD and indicated that PD could be a promising drug for treating rheumatoid arthritis.

However, what are the underlying mechanisms by which PD works in the CIA mouse model? Previous studies have mainly focused on its anti-inflammatory and anti-oxidant effects. Kamel et al. have indicated the anti-arthritis effects of PD could be attributed to its ability for diminishing matrix metalloproteinases such as MMP-3 and RANKL, thus reducing the production of inflammatory cytokines, including IL-6, IL-17, and TNF-α [22]. A study of Bo Li and his colleagues gave another reasonable explanation of the anti-arthritis effects of PD through activating MMP-9 [21].

Meaningfully, a novel and key finding in this study was that PD could significantly inhibit NET formation, providing new evidence to explain how PD protected against collagen-induced arthritis in mice. Our data have clearly revealed that PD could significantly suppress NET formation mediated by neutrophils of RA patients and mice, but also reduce NET deposition in ankle joints of CIA mice. Importantly, increasing evidence has indicated that NET formation plays a crucial role in the pathogenesis and development of rheumatoid arthritis. During NETosis, chromatin and intracellular materials are directly released into the extracellular space, providing multiple autoantigens to the host immune system that induce autoimmune responses and release damage-associated molecular patterns to exacerbate inflammatory reactions [23,24]. In particular, peptidylarginine deiminase (PAD) could mediate the citrullination of exposed proteins during NETosis [25]. Indeed, autoantibodies to citrullinated protein antigens are commonly detected in RA patients. Furthermore, several studies have identified the presence of NETs in skin, rheumatoid nodules, and synovial fluid of RA patients [26]. Similarly, in this study, immunofluorescent staining showed increased NETs in ankle joints of vehicle-treated CIA mice. Carmona-Rivera et al. have indicated that citrullinated peptides of NETs were internalized by fibroblast-like synoviocyte in the synovium and then presented to adaptive immune systems, leading to pathogenic autoimmunity and cartilage damage [27]. Additionally, it has been reported that NETs could induce the maturation of dendritic cells (DCs),

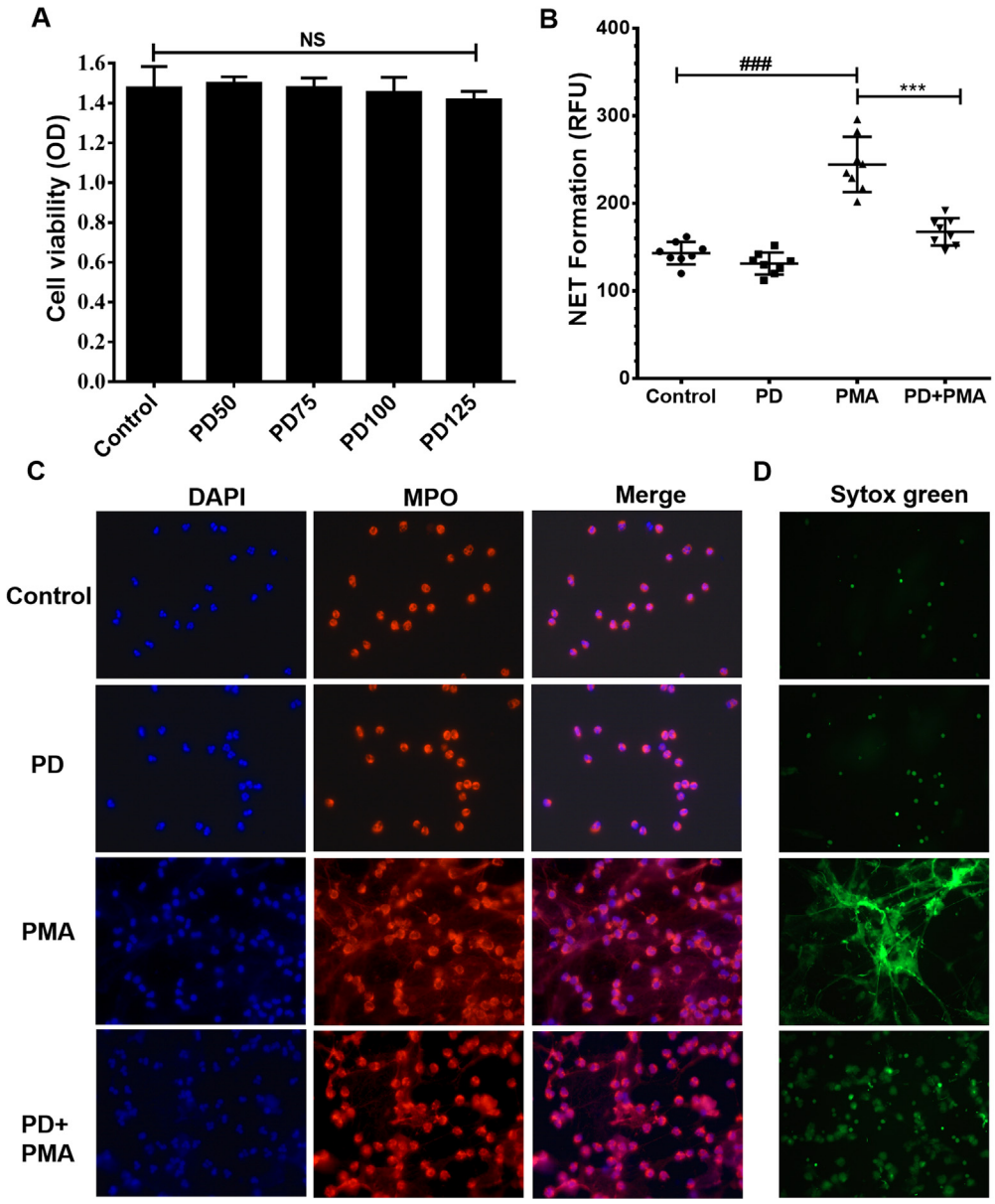

Fig. 1. PD treatment dramatically suppressed PMA-induced NET formation mediated by neutrophils in RA patients in vitro. (A) Effects of PD on cell viability of human neutrophils. Neutrophils were cultured with various concentrations of PD (0, 50, 75, 100 and 125 μg/mL). Cell viability was determined by a CCK-8 assays. (B) In the presence or absence of PD, neutrophils isolated from RA patients were stimulated with PMA for 4 h. NET formation was quantified by SpectraMax M3 Fluorescent Plate Reader, as described in Methods. (C) DNA was stained blue and MPO was stained red. NET formation was consistent with the colocalization of DNA and MPO. Representative fluorescent images of NET formation are shown. (Original magnifcation, ×400). D. SYTOX green was used to stained for DNA and the unfixed NETs were directly imaged with a fluorescence microscope. For all experiments, data were represented as Mean ± SD (n = 8), $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ and were for comparisons between control group and PMA-stimulated group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared the vehicle and PD-treated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

promoting the induction and expansion of Th1 pathogenic cells, as indicated in CIA mouse model [28]. Recently, Lee et al. have indicated that nicotine could induce dose-dependent NET formation and exacerbate inflammatory arthritis, as shown in the CIA model [29]. Altogether, our findings combined with the results of previous studies have demonstrated that PD prevented the development of collage-induced arthritis in mice, probably through inhibiting NET formation.

In conclusion, our data clearly demonstrated that PD significantly inhibited NET formation mediated by neutrophils and effectively protected against arthritis, as shown in the mouse CIA model. These findings uncovered a previously unrecognized mechanism by which PD effectively alleviated arthritic symptoms in CIA mice and provided a solid theoretical basis for the clinical application of PD in treating RA.

Ethics

The experimental contents were approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University.

Acknowledgements

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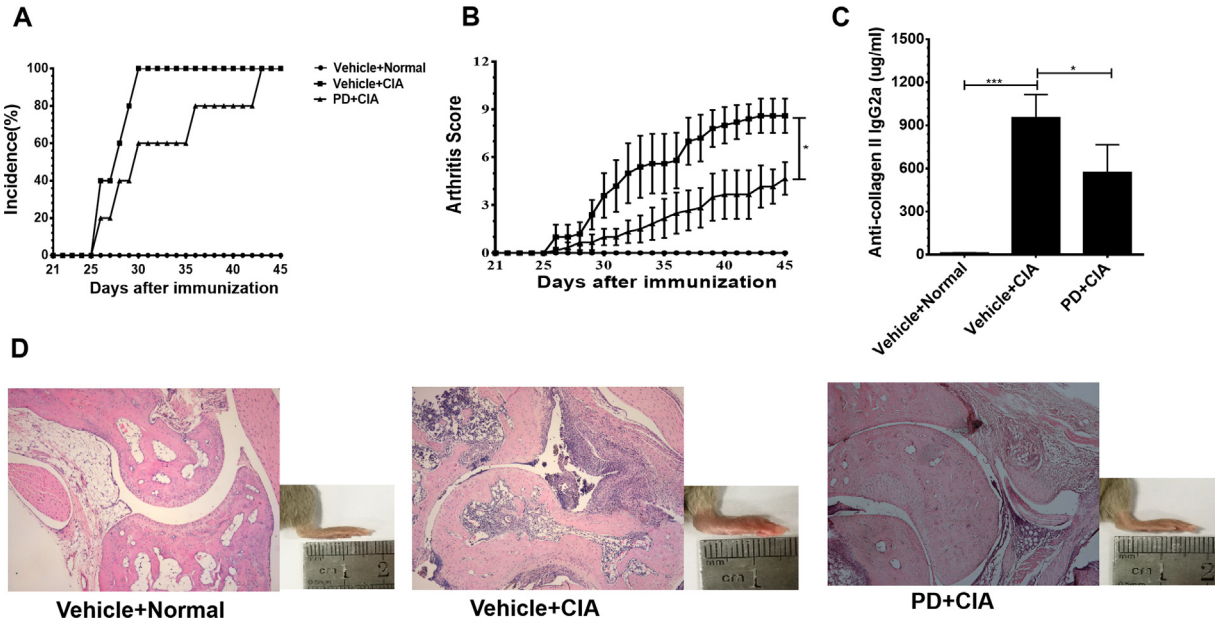

Fig. 2. PD treatment delayed the onset and alleviated the severity of joint arthritis in CIA mice. (A) Arthritis was induced in DBA/1 mice with two injections of collagen type II (CII) emulsion. The collagen-induced arthritis model in mice was successfully established. Therefore, PD treatments could not decrease arthritis incidence, but delayed the onset of arthritis in CIA mice. (B) Compared with the vehicle-treated CIA mice, PD-treated CIA mice showed a significant reduction in arthritis score. (C) The serum levels of anti-collagen II IgG2a antibody in serum of control, vehicle-treated CIA and PD-treated CIA mice. (D) All mice were sacrificed at the end of experiments and joints were collected for further histological analysis. Representative H&E staining of ankle joints was displayed. (Original magnification, ×50). Data were shown as Mean ± SEM (n = 5), *p < 0.05; **p < 0.01; ***p < 0.001.

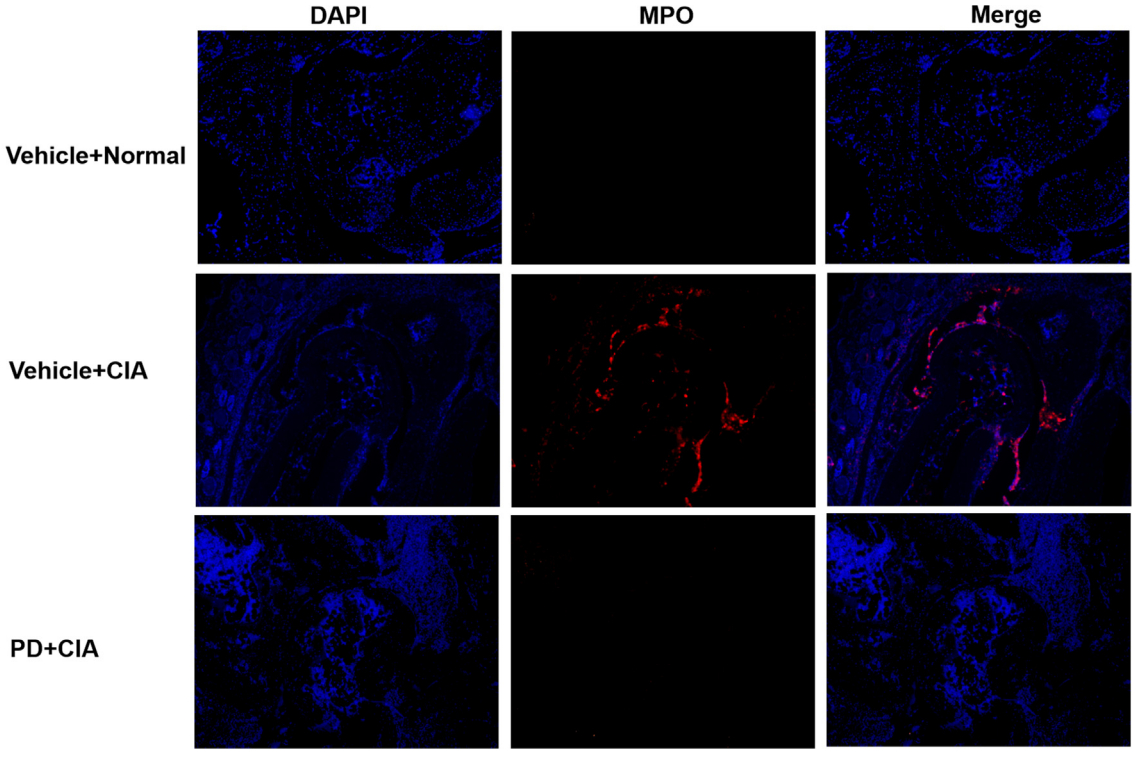

Fig. 3. PD treatment decreased NET formation in inflamed joints of CIA mice. Inflamed joints were stained for the deposition of NETs and the representative staining images are shown above. Abundant NETs in inflamed joints were easily detected in vehicle-treated CIA mice, but rarely found in PD-treated CIA mice.

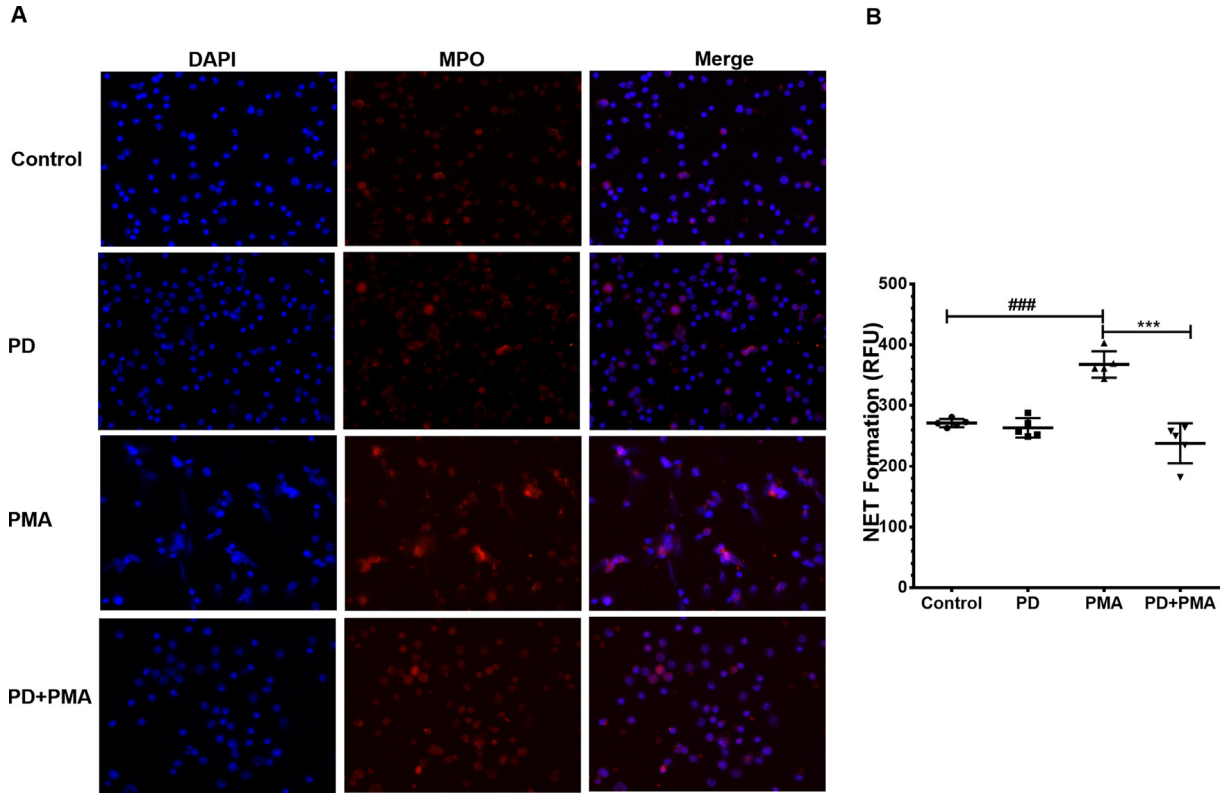

Fig. 4. PD treatment significantly blocked NET release mediated by bone marrow-derived neutrophils in mice in vitro. (A) Bone marrow-derived neutrophils isolated from mice were pretreated with or without PD for 1 h, followed by stimulation with PMA for 4 h. Representative fluorescent images show NET release. (Original magnifcation, ×400). (B) NET formation was quantified as described in Methods. For all experiments, data were plotted as Mean ± SD (n = 5), $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ were for comparisons between control group and PMA-stimulated group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared the vehicle and PD-treated group.

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Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105919.

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